LOCALIZATION OF A TRANSFER RELATED REGION IN STREPTOCOCCAL CONJUGATIVE TRANSPOSON, Tn*5252*

By

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CHAPTER I

INTRODUCTION

In the mid-1970's, multiple antibiotic resistance began to appear in clinical isolates of Gram positive bacteria around the world (6,8,18,20,21,37,42,43). Further investigations revealed that the ability of clinical isolates of Gram positive bacteria to carry resistance to antimicrobial agents was due to mutations in the chromosome or acquisition of extrachromosomal DNA (32). A typical example of the dissemination of multiple antibiotic resistance was observed in *Streptococcus pneumoniae*. This type of resistance even among pneumococci was an unusual phenomenon as this species of bacteria, *S. pneumoniae*, is not known to contain endogenous plasmids (6). These resistances were also observed to be transferred via a conjugation-like process requiring cell-to-cell contact. The biology of this phenomenon has been subject to considerable investigation in the last two decades. This transfer was shown to be due to a novel class of mobile elements termed conjugative transposons (38,43).

A conjugative transposon is a discrete piece of DNA that excises from the donor chromosome and transfers to a

recipient chromosome and inserts without requiring homology. The insertion mechanism of the conjugative transposon is *rec*A independent.

Conjugative Transposons in Streptococci

Many conjugative transposons have been identified in Gram positive bacteria. Localization of Tn*916* in *Enterococcus faecalis* DS16 (8,6,10,31), Tn*1545* in *Streptococcus pneumoniae* BM4200 (28,29), Tn*3701* in *Streptococcus pyogenes* A454 (20,21) and Tn*5253* in *Streptococcus pneumoniae* BM6001 (17,42,43) indicated the dissemination of these elements in streptococci. All these elements have a tetracycline resistance determinant and vary in length from 16 to 60 kb (33).

Previous studies (42,43), have shown that Tn*5253* is a composite transposon (65.5 kb) and has two antibiotic resistance determinants: chloramphenicol (*cat*) and tetracycline (*tet*). The *cat* determinant is flanked by IS-like elements which have been shown to be homologous to the *Staphylococcus* plasmid pC194 (26).

The physical map of Tn*5253* has been constructed by inserting pVA891 at different locations along the element and recovering the vector again in *E. coli* with a passenger DNA from the conjugative element (42,43). Further studies (1) revealed the presence of an independent conjugative transposon within Tn*5253*. This conjugative transposon

carrying tetracycline resistance was named Tn*5251* and the original element, extending beyond Tn*5251* within Tn*5253*, was named Tn*5252*. Tn*5251* is 18 kb in length. Tn*5252* is around 46 kb in length and carries the *cat* determinant (Fig. 1).

Transposition Mechanism

The transposition mechanism of the conjugative transposons is considered a new mechanism in DNA transfer. Although the specific details of the transposition mechanism are still unknown, Clewell et al. (6,10) suggested that in order for the Tn*916* to conjugate intercellularly or intracellularly, the element should excise from the chromosome of the donor cells, form a circular intermediate, transfer from the donor to the recipient cells, and insert into the chromosome of the recipient cells. These four steps are important in conjugative transposition (5,44).

It is believed that the insertion and the excision mechanisms of the conjugative transposons are carried out by Xis and Int proteins (6,28). For example, Tn*1545* is a conjugative transposon in *S. pneumoniae* BM4200 and confers resistance to kanamycin (*aph*A-3), erythromycin (*erm*AM) and tetracycline (*tet*M). This element is 25.3 kb and transfers to a wide variety of Gram positive bacteria. Boyart-Salmeron et al. (28,29) have found that the right side of the Tn*1545* codes for two proteins: Xis-Tn (ORF1) and Int-Tn (ORF2). These proteins were important in the conjugal transfer

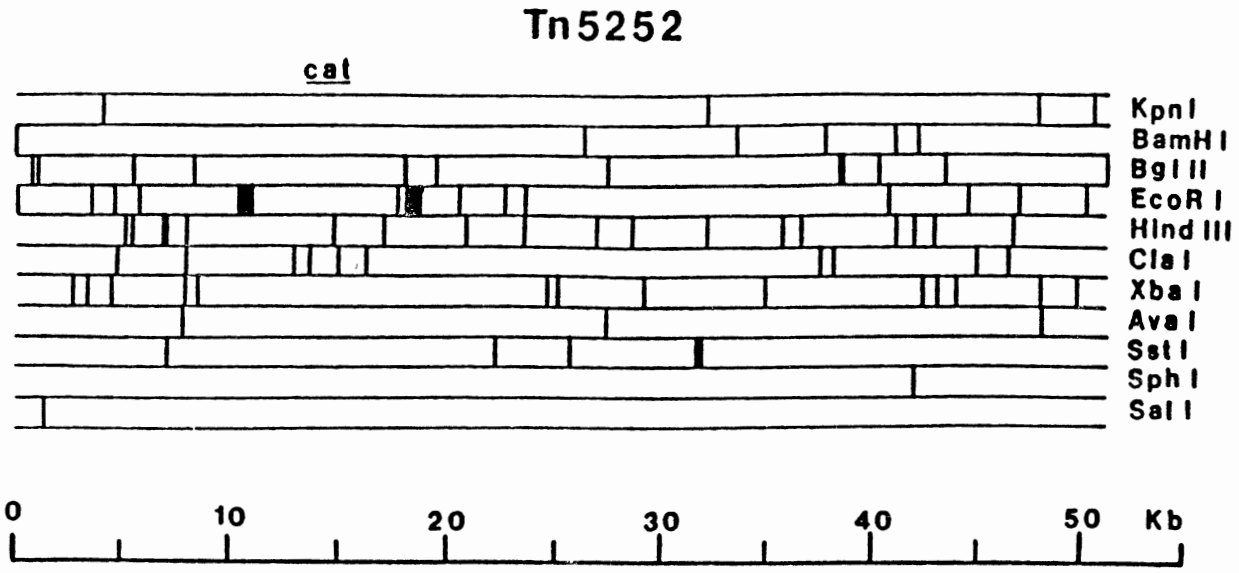

Figure 1. Physical map of Tn*5252* as it is present in SP1000. ▬ IS-like element flanking the *cat* determinant (1,27,28)

of Tn*1545*.

Uniqueness of Conjugative Transposons

One important difference between conjugative transposons (like Tn*916* and Tn*1545*) and other transposons is that the conjugative transposons do not cause duplication of the target sequence upon insertion (33) although it has not been observed in all the conjugative transposons (1). Another difference is that the transposition of conjugative transposons, such as Tn*916* and Tn*1545*, includes a circular intermediate incapable of autonomous replication. In contrast, in non-conjugative transposons the transposition mechanism includes a transient intermediate of the transposon donor and recipient. This transient intermediate is called a cointegrate. For example, Tn*3* encodes for ampicillin and is widespread among Gram negative bacteria (35). The transposition of Tn*3* occurs in two steps. First, transposase protein, coded by TnpA, ligates the donor plasmid to the target sequence in the recipient plasmids in order to form cointegrates. In this step, the selection of the target sequence in the recipient plasmids is random, and the cointegrated molecules are coupled by the replication of the transposon. Second, the cointegrates are resolved by resolvase protein, encoded by TnpR, in order to produce two plasmids in which each plasmid has one copy of the transposon. Therefore, a mutation in the TnpR gene can form

an abundance of the cointegrate molecules in the cell (12,35).

This mechanism was also observed in many Gram positive transposons which are related to the Tn*3* family in forming cointegrate intermediates. These elements are Tn*551* in *Staphylococcus aureus*, Tn*4430* in *Bacillus thuringiensis* and Tn*917* in *E. faecalis* (5,35).

In recent years, it has been suggested that Tn*917* can be used as a genetic tool in introducing insertional mutations in Gram positive bacteria in the same way as Tn*5* in Gram negative bacteria (5,6,19,44,45). Tn*917* is 5.3 kb, confers resistance to erythromycin and was originally identified on a non-conjugative plasmid, pAD2, in *E. faecalis* (5,19). It was found that Tn*917* inactivates the gene upon insertion in *Bacillus subtilis*, *Bacillus megaterium* and other Gram positive bacteria.

Furthermore, Youngman et al. (45) have pointed out that Tn*917* can insert randomly at high frequency in different locations of the chromosome and can be an effective tool to localize genes in Gram positive bacteria. In fact, Youngman et al. have developed many transposon delivery vectors using temperature sensitive plasmids, such as pTV1 plasmids, in order to use Tn*917* as a genetic tool in insertional inactivation mutagenesis. pTV1 variety plasmids are around 12.4 kb and have a low copy number in Gram positive bacteria (around 6 plasmids per cell at 32°C).

These plasmids carry the *cat* determinant on the plasmid vector and *erm* determinant on the Tn*917* transposon.

In order to obtain insertional inactivation mutation using pTV1 plasmid (temperature sensitive replication), it is important to propagate the pTV1 plasmids at a low temperature (32-35°C) and, afterwards, to increase the temperature above 45°C and keep the culture under erythromycin selection at the same time. This will prevent the plasmid from replicating and will induce the Tn*917* insertion at random locations of the chromosome. Soon, the insertion of the Tn*917* will start by forming a cointegrate intermediate and the transient intermediate will be resolved forming two copies of the element, one copy in the recipient DNA and the other copy in the donor DNA. The mechanism of Tn*917* insertion is similar to the Tn*3* family. Furthermore, Camilli et al. (5) used the temperature sensitive plasmids to generate stable insertion mutations in hlyA genes of *Listeria monocytogenesis* (the hlyA gene encodes for a hemolytic protein whose activity is critical in virulence). Using this method, Camilli et al. have been able to insert Tn*917* at eight different locations in the hlyA gene of *Listeria monocytogenesis*.

Localization of the Transfer-related Genes

Many studies have been conducted to localize the transfer-related genes in conjugative transposons. One

effective tool in localizing the transfer-related genes is using Tn*5* mutagenesis (3,4). Tn*5* is a composite transposon, 5.8 kb, flanked by a 1.5 kb terminal inverted repeat (IS50) and it codes for three antibiotic determinants: kanamycin (*kan*), bleomycin (*ble*), and streptomycin (*str*). These three genes are transcribed from the same promoter which is located on the left side of the element. Although the expression of these antibiotic determinants has not been reported in Gram positive bacteria (3), Tn*5* can be used to localize the transfer-related genes of Gram positive transposons when the element is cloned in an *E. coli* plasmid. Tn*5* can create insertional inactivation mutagenesis with a random insertion in the host chromosome.

A typical example of using Tn*5* mutagenesis is to localize the transfer-related genes of the Gram positive conjugative transposon Tn*916*. Tn*916* is 16.4 kb in length, carries *tet*(M) and it is related to many transposons, such as Tn*5251*, Tn*1545*, Tn*3701* and Tn*3951* (1,20,21,28,32). Clewell et al. (5,6,7,8,11,34,44) have found that insertion of Tn*5* in the left side of the Tn*916* prevented both the excision and the insertion of the element in *E. faecalis* indicating that the left side of Tn*916* encodes for two proteins, ORF1 and ORF2. Further studies have shown that the function of these two proteins are similar to the Xis and Int proteins of the lambda phage.

Furthermore, Payart-Salmeron et al. (28,29) used in

vitro deletion mutagenesis to localize the transfer-related genes of Tn*1545*. Using deletion mutagenesis in vitro, Payart-Salmeron et al. cloned the conjugative element in an *E. coli* plasmid and deleted different fragments from within the Tn*1545*. Subsequently, they tested the ability of Tn*1545* to transfer after introducing the element in *S. pneumoniae* again. It was concluded that the left side of the Tn*1545* is involved in the excision of the element.

In this work, a transfer-related region of Tn*5252* was localized by in vivo deletion and ınsertion mutagenesis. We showed that successive interruptions either by insertion or deletion of the 3.39 kb *Xba*I fragment at the left side of the element, Tn*5252*, prevented the element from transferring to *S. pyogenes* and *S. sanguis* in filter-mating experiments ındicating that this region is important in conjugal transposition.

CHAPTER II

MATERIALS AND METHODS

Strains and Plasmids

Rx1 is a non-capsulated wild type *Streptococcus pneumoniae* (6). DP1322 is a Rx1 derivative carrying Tn*5253* (*cat tet*). DP1333 (Tc^s Cm^s tra^+) is DP1322 that has lost chloramphenicol resistance (*cat*) spontaneously and acquired *tet*-1 point mutation conferring tetracycline sensitivity (42). SP1000 is a derivative of DP1322 where 18 kb from the Tn*5253* (*cat tet*) has been deleted in vivo creating Tn*5252* (*cat*) (1). SP1004 is SP1000 that has lost chloramphenicol resistance (*cat*) spontaneously. *Streptococcus pyogenes* ATCC 21545 was provided by Stillwater Medical Center and *Streptococcus sanguis* ATCC 35105 was obtained from Duke University.

All pneumococcal strains are listed in Table 1 and the chromosomal genetic markers mutations in streptococcal strains are listed in Table 2. The phenotype and the genotype of the antibiotic resistant markers are listed in Table 3.

In constructing new recombinant plasmids, we used three

E. coli recombination deficient strains: DH5α, DH1 and JM109. pVA891 is a recombinant plasmid which can replicate only in *E. coli* (22,42). The plasmid has two antibiotic resistance determinants: chloramphenicol and erythromycin. Both of the antibiotic determinants can be expressed in *E. coli*, but only erythromycin can be expressed in pneumococcus.

pSS141 is a recombinant plasmid containing pVA891 as a vector and a 21 kb *Kpn*I fragment from the left side of Tn*5252* without the *cat* region. All the *E. coli* strains and plasmids used in this study are listed in Table 4.

Media and Buffers

All pneumococcal strains were grown in CAT medium. The content of CAT medium was 10 g of casitone, 5 g of tryptone, 1 g of yeast extract per liter of dH_2O. This mixture was autoclaved for 20 minutes. Subsequently, 1/30 (v/v) of 0.5 M K_2HPO_4 and 1/100 (v/v) of 20% glucose were supplemented aseptically when the medium cooled down to 45°C. For solid CAT medium, 1.5 g of agar was added to 100 ml of the medium before autoclaving.

CTM-D10 media used to harvest the conjugation mixture contains CAT medium supplemented with 10% (v/v) glycerol, BSA (2 mg/ml), 10 mM $MgSO_4$ and DNase I (50 μg/ml).

Luria-Bertani (LB) medium used to grow *E. coli* was prepared according to Maniatis (23). *E. coli* cells were

TABLE 1

Streptococcus pneumoniae STRAINS

Strain	Genotype	Reference
Rx1	*hex*	(24)
DP1333	Tn*5253* (⌂ *cat tet*-3)	(26)
SP1000	*str*-1 *fus* Tn*5252* (*cat*)	(1)
SP1004	*str*-1 Tn*5252* (⌂ *cat*)	This study
DP1004	*str*-1	(12)
DP1617	*hex*$^+$ *str*-1 *ery*-2 *nov*-1 *fus salf*-d *stg*	(26)
GP42	*str*-1 Tn*5253* Em^r (⌂ *tet erm*)	(27)
SP1014	Tn*5253* Em^r(⌂ *cat tet*-3 *erm*)	This study
SP1015	*str*-1 *fus* Tn*5252* Em^r (*cat erm*)	This study
SP1018	*str*-1 *fus* Tn*5252* Cm^r (⌂ 23 kb *Hin*dIII fragment *cat*)	This study
SP1019	*str*-1 *fus* Tn*5252* Em^r (⌂ 3.22 kb *Cla*I fragment, ⌂ *cat erm*)	This study

hex$^+$ Cells which have a mismatch correction system at the heteroduplex stage of transformation.

TABLE 2

CHROMOSOMAL GENETIC MARKERS[a]

Genotype	Phenotype	Selection
nov-1	Nov^r	novobiocin
str-1	Str^r	streptomycin
ery-1	Ery^r	erythromycin
fus	Fus^r	fusidic acid
sul-d	Sulf^r	sulfanilamide
stg	Stg^r	streptolidigin

a These are point mutations in the chromosome conferring the described phenotype.

TABLE 3

ANTIBIOTIC RESISTANT MARKERS

Genotype	Phenotype	Antibiotic marker
cat	Cm^r	chloramphenicol acetyl transferase
tet	Tc^r	tetracycline M resistance
amp	Amp^r	ampicillin
erm	Em^r	erythromycin

TABLE 4

E. coli STRAINS AND CLONING VECTORS

Strain	Genotype	Source
DH5α	F⁻ recAI	Bethesda Lab.
DH1	F⁻ recAI	Bethesda Lab.
JM109	*rec*A1	Bethesda Lab.
Plasmids	**Sizes and Markers**	
pVA891	5.88 kb, Cm^r Em^r	(15,27)
pSS141	26.2 kb, Cm^r Em^r	This study
pVJ438	pU18 (*Xba*I)::3.3 kb *Xba*I fragment of Tn*5252* amp^r lac$^-$	This study
pVG85	pMV185 (*Hin*dIII):: 2.2 kb *Hin*dIII (cat cassette) of Tn*5252*	This study

grown in SOB medium prepared according to Maniatis (23) prior to transformation.

Many common buffers were used in this study such as: SSC (0.15 M NaCl 0.015 M sodium citrate), TE (10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0)), TBE (0.089 M Tris base, 0.089 M boric acid, 2.5 mM EDTA, pH 8.3).

Growth and Storage of Bacterial Strains

All pneumococci and *Streptococcus pyogenes* strains were grown in CAT medium up to 2 x 10^8 CFU/ml and were kept in 10% (v/v) glycerol at - 80°C for further use.

E. coli strains were grown overnight in LB with aeration and the strains were kept in 50% (v/v) glycerol at - 20°C for further use. The antibiotic concentrations used for different strains are shown in Table 5.

Enzymes and Chemicals

T4 DNA ligase, restriction endonucleases and the molecular weight standard (lambda DNA digested with *Hin*dIII and/or *Hae*III) were purchased from either Bio-Rad Laboratories (BRL) or United States Biochemical Company (USBC). DNase I, RNase I and egg white lysozyme grade 1 were purchased from Sigma or USBC. Media and agar for bacteria were purchased from Difco or USBC. Agarose for gel electrophoresis was purchased from Fisher or International Biotechnologies, Inc. For electroeluting DNA fragment after

TABLE 5

SELECTIVE ANTIBIOTIC CONCENTRATION

Antibiotic	Concentration (μg/ml) Stab plate/broth	overlay
Streptococcus pneumoniae		
chloramphenicol (Tn*5253*)	5	15
erythromycin	3	5
fusidic acid	10	50
novobiocin	10	10
rifampicin	10	20
streptomycin	200	200
tetracycline (Tn*5253*)	2	5
Streptococcus pyogenes		
chloramphenicol	5	
streptomycin	200	
erythromycin	5	
optochin	20	
Streptococcus sanguis		
chloramphenicol	5	5
streptomycin	200	1000
erythromycin	5	10
tetracycline	5	10
optochin	20	20
Escherichia coli		
ampicillin	40	
chloramphenicol	10	
erythromycin	200	
tetracycline	10	

electrophoresis, Ultrapure agarose from Bio-Rad Laboratories was used. [α-^{32}P] dCTP was purchased from New England Nuclear.

Isolation of Genomic DNA

Streptococcus pneumoniae cultures were grown in 250 ml of CAT medium at 37°C until the culture reached 2 x 10^8 CFU/ml. Subsequently, 5 ml of 0.5 M EDTA was added to the culture at 0°C. All the cells were harvested in 250 ml polypropylene bottles in a Sorvall GSA rotor at 7,000 RPM at 4°C for 10 min. The pellet was washed in 20 ml of 1x SSC (0.15 M NaCl, 0.015 M sodium citrate) and the cells were harvested by centrifugation again.

The pellet was resuspended in 3 ml of 1x SSC and the following were added sequentially: 5 μl of diethyl pyrocarbonate, 200 μl of 0.3 M EDTA, 40 μl of 0.1% (w/v) sodium dodocyl sulfate (SDS) and 200 μl of 0.1% (w/v) sodium deoxycholate (DOC). The cell suspension was kept at 37°C until cells lysis (this usually takes 15-20 minutes). The lysate was used to prepare purified DNA according to Marmur (24).

Preparation of Plasmid DNA

E. coli strains were grown in L-broth overnight with aeration (stationary phase). The plasmid was isolated according to standard methods involving lysozyme treatment.

Subsequently, the plasmid was extracted with phenol-chlorpform-isoamyl alcohol mixture (25:24:1) following sedimentating the DNA in a cesium chloride-ethidium bromide density gradient. Small-scale preparation of the plasmid was performed by using an alkaline lysis method as recommended by Maniatis (23).

Agarose Gel Electrophoresis

Gel electrophoresis of DNA was conducted in a horizontal agarose gel at room temperature in 1/2x TBE and the concentration of the agarose was 0.6-0.8%. DNA restriction fragments for subcloning were isolated by electroelution after electrophoresis in a Bio-Rad Ultrapure agarose gel. The DNA fragments were extracted once with a phenol-chloroform-isoamyl alcohol mixture (25:24:1), extracted twice with chloroform-isoamyl-alcohol (24:1) and precipitated with two volumes of 100% ethanol. The DNA was resuspended in the TE buffer.

Transformation

Frozen competent pneumococcal strains in - 80°C were used in transformation. Transformation and the plating techniques were done as described in by Guild shoemaker (13). *E. coli* transformation was done according to Hanahan (34). The cells were plated with the appropriate selective antibiotic.

Filter-mating

Prior to all filter-mating experiments, pneumococcal strains and *Streptococcus pyogenes* cells were grown in CAT medium containing Difco Bacto-casitone. *Streptococcus sanguis* was grown in BHI overnight and diluted two fold before mixing it with the *Streptococcus pneumoniae* donors.

The donor and the recipient cells were mixed at a ratio of 1:5 (donor:recipient) in the presence of DNase I (50 μg/ml), BSA (2 mg/ml) and 10 Mm $MgSO_4$. The cell suspension was filtered through sterile 13-mm nitrocellulose filters. Then the filter was placed cell-side-down on CAT agar containing DNase I (50 μg/ml), BSA (2 mg/ml) and 10 Mm $MgSO_4$. The filter was overlaid with the same agar as in the base agar and incubated for 4 hours at 37°C. After the incubation period, the filter and the surrounding agar were harvested in CTM-D10 media (see media and buffers). Consequently, the conjugation mixture was plated on the surface of CAT medium plates containing the appropriate selective antibiotics to select for transconjugants.

The phenotype of the transconjugants was confirmed by replica plating with a sterile toothpick onto the appropriate selective media containing 2% bovine blood.

DNA-DNA Hybridization

The conditions of labeling the two probes (pSS141 and

1.34 kb *Hin*dII fragment of Tn*5252*), nick translation, were done according to the standard methods using [α-^{32}P]dCTP. Denaturing, transferring the DNA from the gel to the membrane (Gene Screen Plus), prehybridization and hybridization were done according to the manufacturer's instructions (New England Nuclear) using a vacuum transfer apparatus.

CHAPTER III

RESULTS

Recent studies have provided evidence that antibiotic resistance determinants of *Streptococcus pneumoniae* transfer in the absence of a detectable plasmid by a conjugation-like process. In further work (8,37), it was reported that Cm^r Tc^s transformants or Cm^s Tc^r transformants recovered upon transforming RxI with DNA isolated from *S. pneumoniae* BM6001 were unable to transconjugate in filter-mating experiments. Whereas, Cm^r Tc^r transformants were able to transconjugate to RxI in a filter-mating experiment (8,30). This suggested that the two markers were linked in a discrete piece of DNA. This discrete piece of DNA is now termed Tn*5253*.

Tn*5253* is 65.5 kb in length and in vivo deletion of the 18 kb segment, Tn*5251*, from the conjugative element did not affect the conjugal transfer of the remaining sequence, Tn*5252* (1). Tn*5252* transfers by filter-mating to Rx1 at a frequency of 10^{-6} to 10^{-7} per donor. It was also observed that Tn*5253* was capable of conjugal transfer without a 7.5 kb-*cat* segment to RxI in filter-mating experiments. This indicated that Tn*5251* and the 7.5 kb-*cat* segment did not affect the conjugal transfer of the larger element Tn*5253*.

Therefore, localizing the transfer-related genes within Tn*5252* might provide further insight into the nature of its conjugative transposition.

One method of localizing the transfer-related gene(s) in Tn*5252* is Tn*917* insertional inactivation mutagenesis. This transposon can insert randomly at different locations in Tn*5252* creating a variety of mutants. Then, these mutants can be tested for the conjugal transfer ability of the element and the *tra*$^-$ mutants can be identified. Afterwards, by using Southern hybridization to localize the precise location of Tn*917* insertion, the possible location of the transfer-related gene(s) in Tn*5252* can be identified. One difficulty which might be encountered in this method is the tedious work of conducting many filter-mating experiments to identify the *tra*$^-$ mutants. Therefore, an alternate method was used to localize the transfer-related region of Tn*5252*.

In this work, directed deletion mutagenesis within Tn*5252* was used to create *tra*$^-$ mutants. If the mutant is *tra*$^-$, a smaller fragment would be deleted within the original deleted region for further localization of the transfer-related region. If the smaller fragment deletion mutant was *tra*$^-$ as well, insertional inactivation mutagenesis would be conducted to localize the precise region of transfer-related gene(s). This would be done by inserting pVA891 within the element. This method was found

to be successful and it was possible to identify one region that is important in the conjugal transfer of Tn*5252*.

To carry out this goal, it was important to construct a recombinant plasmid carrying a large fragment from the element Tn*5252*.

Directed Insertion of a Plasmid Vector

A strategy for cloning a piece of DNA of the conjugative element was used by selecting a vector carrying markers which can be expressed in both *S. pneumoniae* and *E. coli*. This vector was pVA891 which has two markers, chloramphenicol and erythromycin resistances. These two markers are expressed in *E. coli* but only erythromycin can be expressed in *S. pneumoniae*. pVA891 is capable of autonomous replication in *E. coli*, but it can not replicate autonomously in pneumococcus.

This property of pVA891 allowed us to insert the vector in the conjugative element of *S. pneumoniae* and recover different fragments in *E. coli* by plating the transformants on plates containing chloramphenicol.

Construction of SP1014.

To direct the insertion of the vector plasmid, pVA891, within the conjugative element, the 3.39 kb *Xba*I fragment from the left side of Tn*5252* (coordinate 4.8-8) was cloned into the *Xba*I site of pUC18 in order to create a recombinant

plasmid, pVJ438 (Vijayakumar). pVJ438 is *lac*⁻ and confers resistance to ampicillin in *E. coli*. pVJ438 has a unique *Bgl*II site present within the *Xba*I cloned fragment of Tn*5252*. pVJ438 was digested with *Bgl*II restriction endonuclease and ligated to pVA891 digested with *Bam*HI restriction endonuclease (Fig. 2a).

Before transformation of SP1000 carrying Tn*5252* and DP1333 carrying Tn*5253* (⌂ *cat tet*-3), the ligated molecule (pVJ438-*Bgl*II:pVA891-*Bam*HI) was digested with *Kpn*I restriction endonuclease in order to create a linear pVA891 flanked by the *Xba*I fragment (Fig. 2b); the *Kpn*I site occurs in the multiple cloning site of vector pUC18. It was expected that the donor linear molecule will be flanked by homologous DNA that will pair to the conjugative element. This pairing will form a heteroduplex with a single-stranded loop which represents pVA891 (Fig. 2c). Thus, after one round of replication, the heteroduplex will be resolved producing two daughter cells, one of them carrying pVA891. It should be noted that this mechanism of integration could be used to produce either an insertion or a deletion (39).

Transformants were selected by plating the mixture on plates containing erythromycin and the transformation results are given in Table 6. Unfortunately, Em^r transformants were only recovered from DP1333. One of these transformants was termed SP1014 (Fig. 3). The inability to obtain Em^r transformants from SP1000 and the low

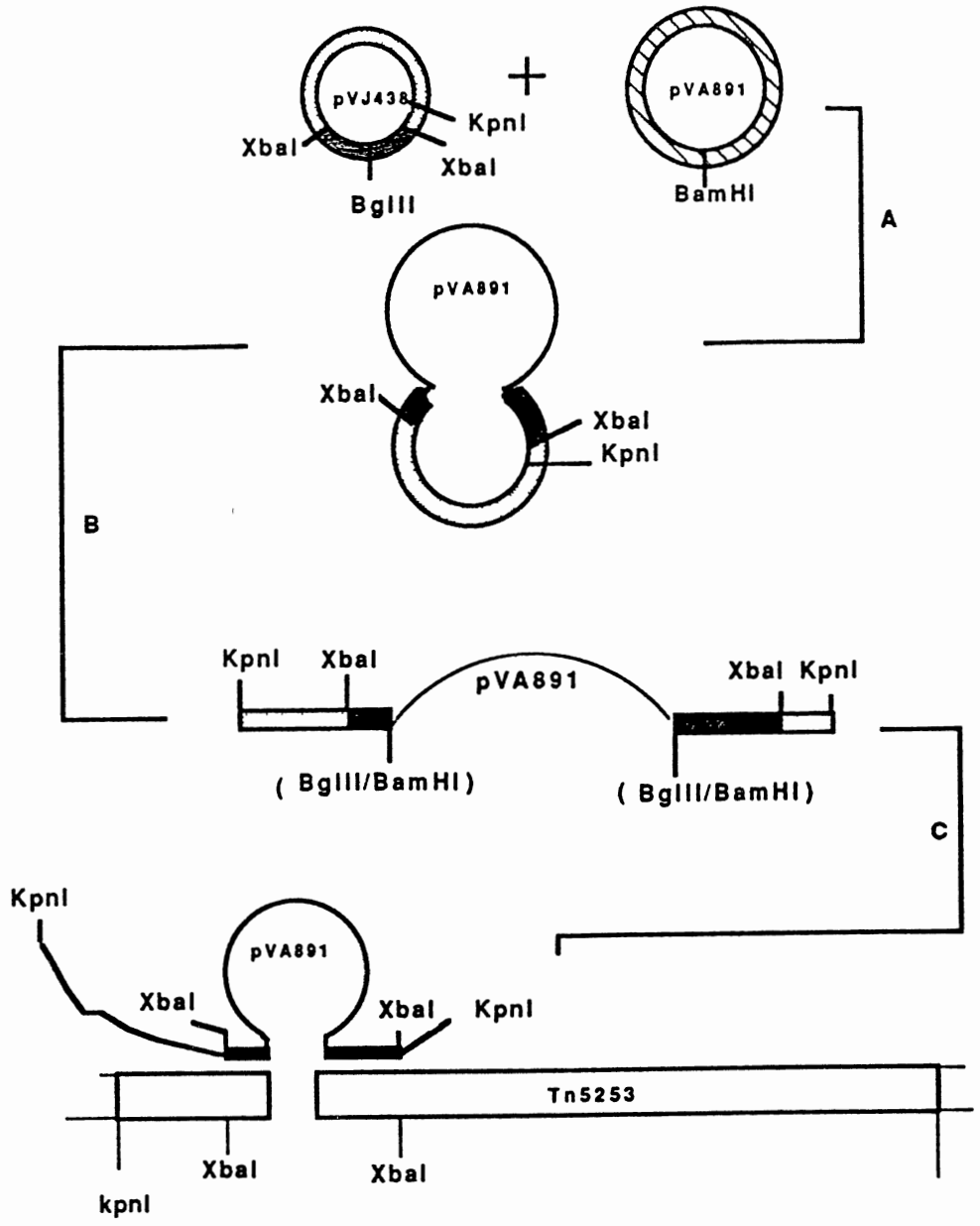

Figure 2. Construction of SP1014. (a) The cloning of *Bam*HI digested vector pVA891 into the *Bgl*II site of *Xba*I fragment of pVJ438. (b) Digesting the ligated molecule (pVJ438-*Bgl*II:pVA891-*Bam*HI) with *Kpn*I restriction endonuclease. (c) Transforming the *Kpn*I digested molecule (pVJ438-*Bgl*II:pVA891-*Bam*HI) to pneumococcus competent cells where a single strand of pVA891 flanked by *Xba*I fragment is incorporated into *Bgl*II of the conjugative element.

TABLE 6

TRANSFORMATION OF *S. pneumoniae* CARRYING THE CONJUGATIVE ELEMENT USING THE CHIMERIC MOLECULE (pVJ438-*Bgl*II:pVA891-*Bam*HI) DIGESTED WITH *Kpn*I

Donor DNA	Recipient Cell	Selection	Transf/ml
pVJ438/pVA891[a]	DP1333	Em 5 μg/ml	35
pVJ438/pVA891[a]	SP1000	Em 5 μg/ml	<5
DP1617[b]	DP1333	Str 200 μg/ml	1.2×10^5

a pVJ438-*Bgl*II:pVA891-*Bam*HI molecule was digested with *Kpn*I restriction endonuclease before transforming DP1333 and SP1000.

b DP1617 DNA was used as a control to measure the transformation frequency of DP1333.

transformation frequency of Em^r transformants DP1333 (35 transformants/ml) were expected because such an event was expected to be rare due to the nature of the transfer of a long strand (9 kb) without interruption (27).

In further work, SP1014 chromosomal DNA was used to recover the pVA891 vector with different fragments from the conjugative element. The SP1014 chromosomal DNA was used also to create SP1015 mutant where the vector is inserted in Tn*5252*.

Construction of pSS141 in *E. coli*.

Different fragments from Tn*5253* were recovered carrying pVA891 as a vector in *E. coli*. Chromosomal DNA of SP1014 with pVA891 inserted at the *Bgl*II site of Tn*5253* was isolated, digested with different restriction endonucleases, self-ligated and then introduced into *E. coli* DH1 (Table 7). Cm^r transformants were ısolated and screened for plasmids by agarose gel electrophoresis.

Furthermore, only the plasmid resulting from *Kpn*I restriction endonuclease digestion was selected for further study. This plasmid was found to have 21 kb of the *Kpn*I fragment (without the cat regıon) from the left side of the Tn*5253* (⌂ *cat*, *tet*-3) as passenger DNA. The physical measurements of the plasmid ındicated that the vector has indeed inserted in the *Bgl*II site of Tn*5253* and that the restriction endonuclease of the physical map of the

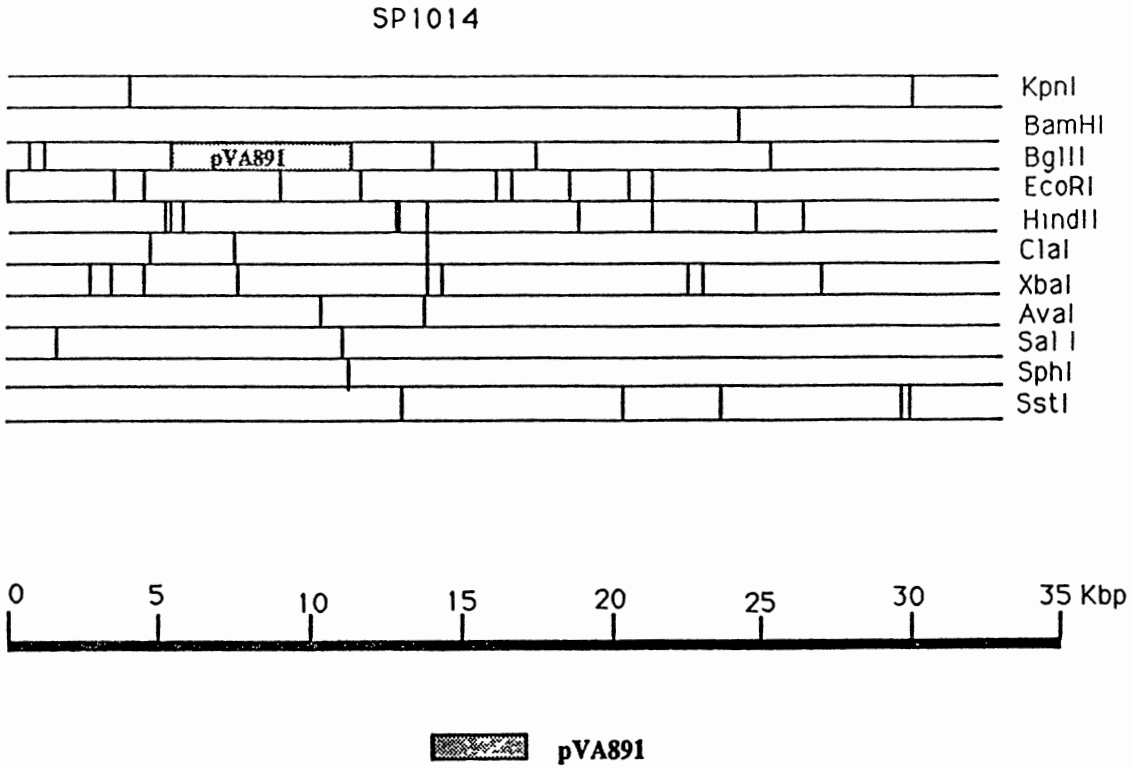

Figure 3. Physical map of the left region of Tn*5253* as it is present in SP1014. pVA891 was inserted at the *Bgl*II site of the left side of Tn*5253* in DP1333 (Tn*5253* ∆ *cat*, *tet*-3) by flanking homology in order to create SP1014.

TABLE 7

TRANSFORMATION OF *E. coli* DH1 WITH DIGESTED PNEUMOCOCCAL DNA OF SP1014

DNA	Endonuclease Digestion and Self-ligation	Transformants/ml[a]
SP1014[b]	*Kpn*I	1×10^2
	*Sal*I	1×10^3
	*Bgl*II	3.2×10^3
	*Bam*HI	1.8×10^3
	none	<5
pVA891[c]	none	2.1×10^5

a The selection was always chloramphenicol 10 μg/ml.

b SP1014 chromosomal DNA was digested with the listed restriction endonucleases and self-ligated in the appropriate conditions before transforming *E. coli*.

c pVA891 transformation was used as a control to detect the transformation frequency of the *E. coli* competent cells.

passenger DNA corresponded with the original physical map of the *Kpn*I fragment of Tn*5253* (42). This plasmid was termed pSS141 and the physical map is presented in Figure 4.

Fusion fragments of pSS141 of different restriction endonucleases which represented the ligation of the two ends of the *Kpn*I termini were used in directed deletion mutagenesis within Tn*5252*.

Directed Deletion Mutagenesis

pSS141 recombinant plasmid was used as a source of fusion fragments containing a unique *kpn*I site to introduce directed deletion mutagenesis in vivo. The 23 kb *Hin*dIII segment and 3.22 kb *Cla*I fragment from the left side of Tn*5252* were deleted. These deletion mutants were confirmed by Southern hybridization using different probes and tested for their conjugal transfer of the element by filter-mating experiments.

Deletion of a 23 kb *Hin*dIII Fragment from within Tn*5252* and Construction of SP1018

pVG85 is 7.4 kb and it was originally created by cloning a 2.2 kb *Hin*dIII fragment that contains the *cat* region of Tn*5252* in the *Hin*dIII site of pMV158 (Vijayakumar,40,42). In order to carry out the deletion of the 23 kb *Hin*dIII segment flanked by *Hin*dIII sites from within the left side of Tn*5252*, a 4.6 kb *Hin*dIII fusion

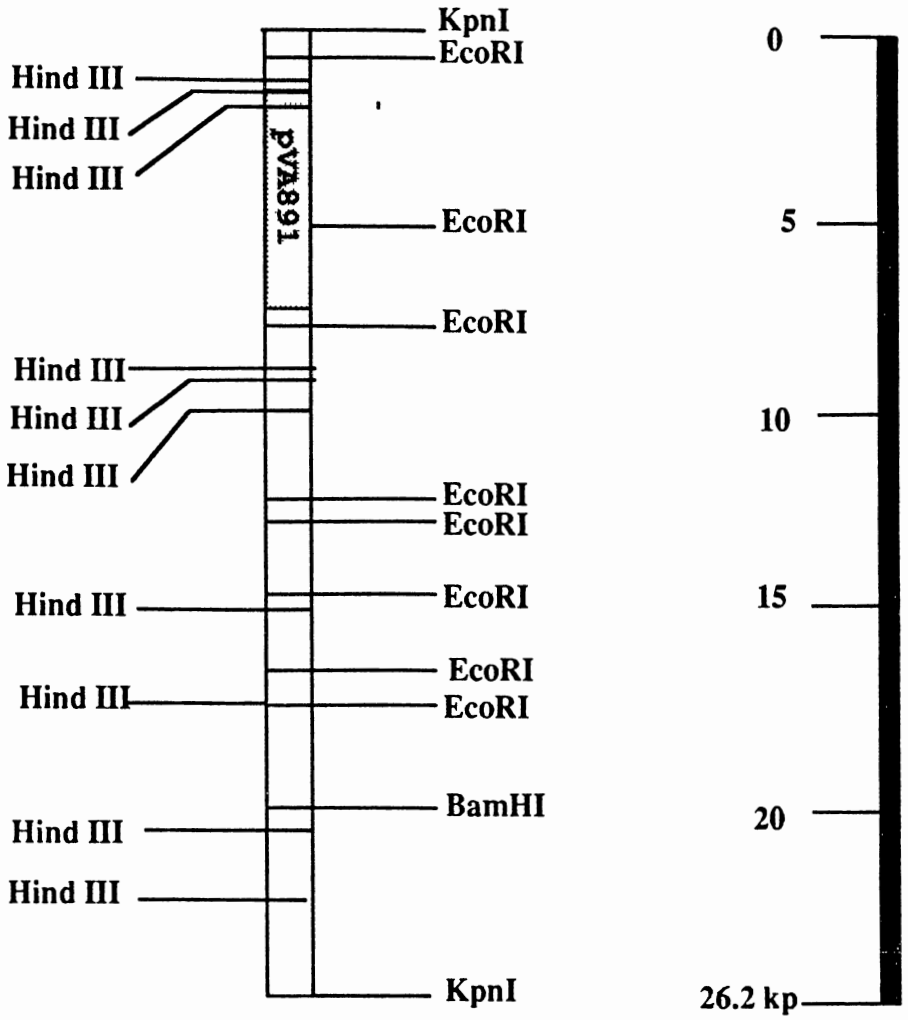

Figure 4. Restriction endonuclease map of pSS141, generated in *E. coli* DH1 from *Kpn*I restriction endonuclease digest of SP1014 DNA and self-ligated. The vector pVA891 is indicated by ▭ which is inserted by flanking homology in the *Bgl*II site of the passenger DNA.

fragment containing the *kpn*I site of pSS141 was electroeluted from pSS141 and ligated to the 2.2 kb *Hin*dIII fragment carrying the *cat* gene of pVG85. Furthermore, the ligated molecule (the fusion fragment and 2.2 kb *Hin*dIII fragment) was digested again with *Kpn*I restriction endonuclease to linearize the DNA molecule. The linear molecule was added to competent cells of SP1004, and the selection of the transformants that contain the deletion was done by plating the transformants on media containing chloramphenicol.

The transformation frequency is listed in Table 8. The number of transformants as it appears in Table 8 is low (50 transformants/ml) compared to the control. This low frequency of transformation was due to the low possibility of the entry of a long single-stranded DNA without interruption in order to produce a 23 kb deletion from within the element.

Deletion of a 3.22 kb *Cla*I Fragment from within Tn*5252* and Construction of SP1019

A 16.9 kb *Cla*I fusion fragment containing the *kpn*I site of pSS141 was electroeluted and ligated to pVA891 which was digested with *Cla*I restriction endonuclease. The ligated mixture was digested by the *Kpn*I restriction endonuclease again to create a linear molecule, and the linear molecule was added to SP1004 (Tn*5252* ⌂ *cat*) competent cells (Fig. 5).

TABLE 8

TRANSFORMATION OF SP1004 COMPETENT CELLS WITH *Hin*dIII DIGESTED 4.6 kb FUSION FRAGMENT[a]

DNA	Selection	Transformants/ml
4.7 kb *Hin*dIII[a]	Cm 5 μg/ml	50
DP1617[b]	Nov 10 μg/ml	1.8×10^6

a 4.6 kb *Hin*dIII fusion fragment containing the *kpn*I site of pSS141 was ligated to 2.2 kb *Hin*dIII fragment of pVG85 which contains the *cat* cassette and digested with *Kpn*I restriction endonuclease before transforming SP1004.

b DP1617 was used as control to measure the transformation frequency of SP1004.

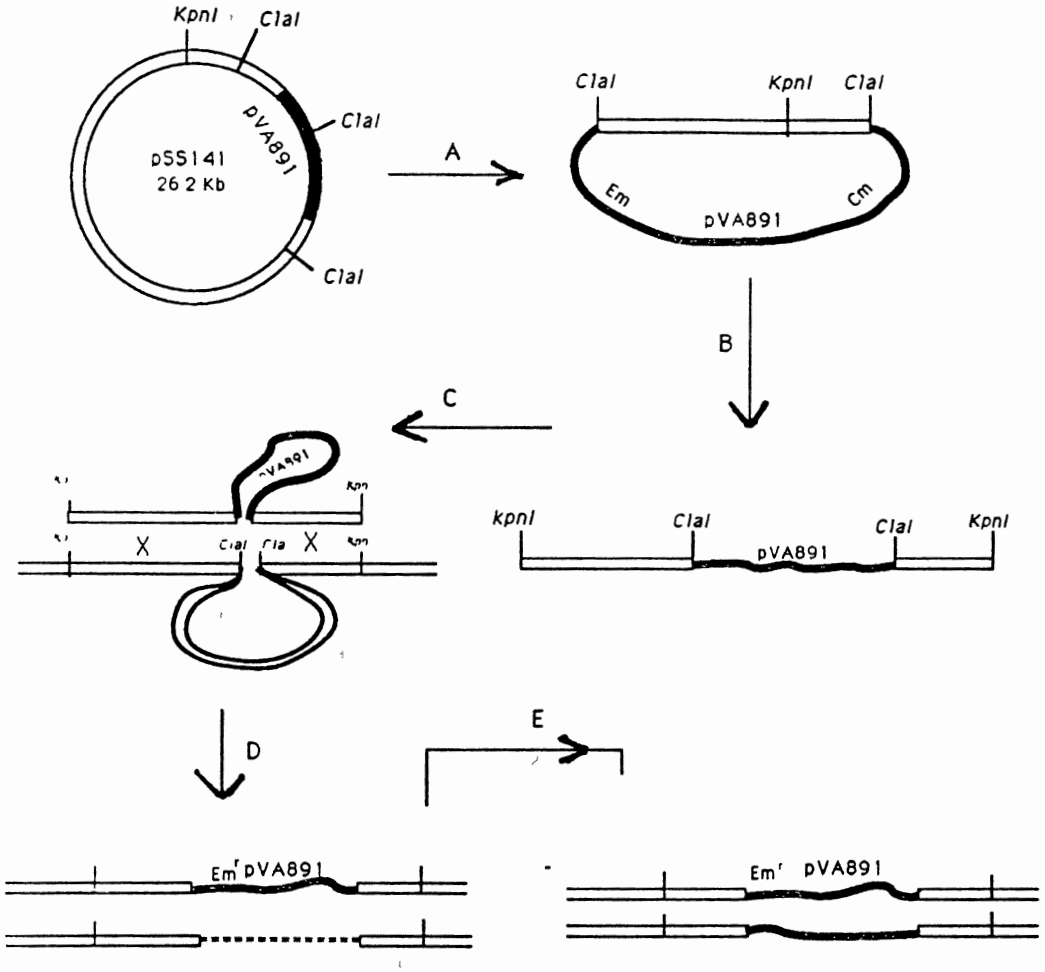

Figure 5. Site directed-deletion mutagenesis of the 3.22 kb *Cla*I fragment from within Tn*5252* (a) Ligating the 16.9 *Cla*I fragment to *Cla*I-pVA891 (b) Linearizing the molecule with *Kpn*I restriction endonuclease (c) Transforming the molecule to SP1004 (Tn*5252* ⌂ *cat*) (d) Replacing the SP1014 DNA fragment with a non-homologous DNA fragment (pVA891). Creating the deletion mutant. (e) SP1019.

The transformants were selected on plates containing erythromycin. Upon transformation, the vector pVA891 replaced the 3.22 kb *Cla*I fragment (coordinate between 4.8-8) of Tn*5252*. The transformation frequency is given in Table 9. The transformants were replica plated and observed to be Em^r Str^r. One of these transformants was termed SP1019.

Figure 6 indicates the deletion mutant of SP1018 and SP1019 with precise coordinates in the original partial physical map of Tn*5252*.

Creation of Insertional Inactivation Mutant SP1015

We predicted that inserting pVA891 in the *Bgl*II site by flanking homology in Tn*5253* (SP1014) might have an effect on the conjugal transfer of the element Tn*5252*. Furthermore, the chromosomal DNA from SP1014 was isolated, diluted, needle-sheared, and added to SP1000 competent cells carrying Tn*5252*. The transformation mixture was plated on media containing erythromycin (Data not shown). Ten transformants were ısolated and one of them termed SP1015. Shearing the DNA of SP1014 was to prevent a long donor single strand from transformıng SP1000 and to allow only the vector which is flanked by sufficient homology for insertion into Tn*5252* of SP1000. These transformants were replica

TABLE 9

TRANSFORMATION OF SP1004 COMPETENT CELLS WITH *Cla*I DIGESTED 16.9 kb DNA FUSION FRAGMENT[a]

DNA	Selection	Transformants/ml
16.9 kb *Cla*I[a]	Em 5 μg/ml	5
DP1617	Nov 10 μg/ml	1.2×10^6

a The 16.9 kb *Cla*I fusion fragment was ligated to *Cla*I digested vector pVA891 and the ligated molecule was digested again with *Kpn*I restriction endonuclease before transforming SP1004 (Tn*5252* ⌂ *cat*).

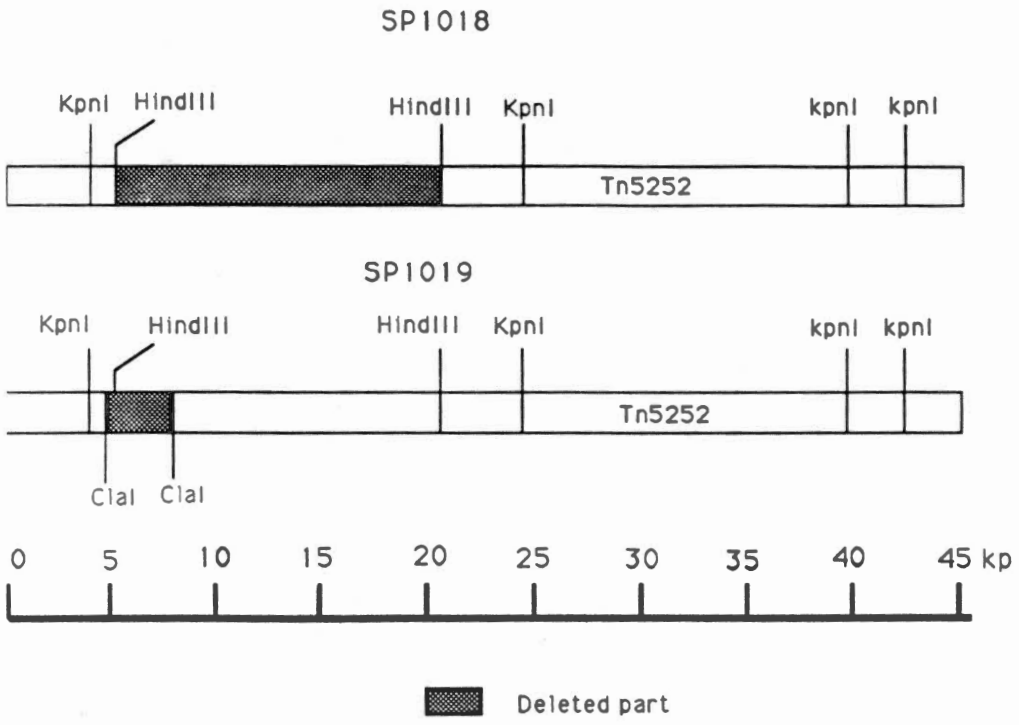

Figure 6. Partial physical map of the deletion regions of SP1018 and SP1019. (a) SP1018: 23 kb *Hin*dIII fragment deleted from within Tn*5252* and replaced with 2.2 kb *Hin*dIII cat cassette. (b) SP1019: 3.22 kb *Cla*I fragment deleted from within Tn*5252* and replaced with pVA891.

plated and were observed to be Em^r Cm^r Str^r. This insertional inactivation mutant strain SP1015 was used in filter-mating experiments. Figure 7 shows the creation of insertion and deletion mutant strains.

Physical Analysis of the SP1018 and SP1019 Deletion Mutants by Southern Hybridization

In order to confirm the two deletions from within Tn*5252* in SP1018 and SP1019, Southern hybridization was used. Chromosomal DNA from the controls (RxI, SP1000, and DP1322), and the mutants, SP1018 and SP1019, were isolated, digested with *Eco*RI and *Hin*dIII restriction endonucleases, separated on an agarose gel, transferred to a nitrocellulose filter membrane, and probed separately with ^{32}P-labeled pSS141 and internal 1.34 kb *Hin*dIII fragment (coordinate 5.5-6.9) of Tn*5252*.

Confirmation of the Deletion of 23 kb *Hin*dIII Fragment from within Tn*5252* in SP1018 by Southern Hybridization

If the 23 kb *Hin*dIII fragment is deleted from within Tn*5252* in the SP1018 mutant strain, the ^{32}P-labeled pSS141 will react to the control strains, SP1000 and DP1322, and it will not react to any fragment within the 23 kb *Hin*dIII deleted region. This will be observed when the pSS141 probe reacts to seven *Eco*RI fragments of the SP1000 control

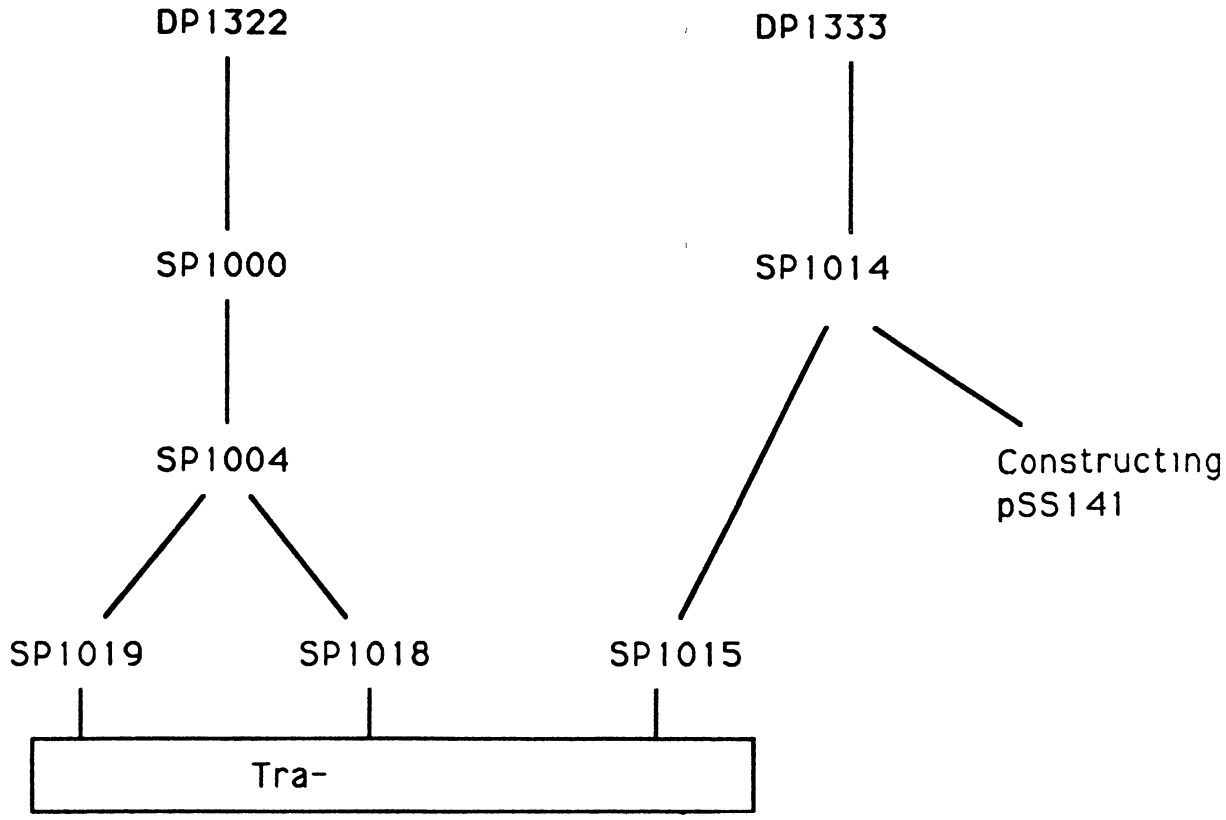

Figure 7. Flow chart showing the creation of the insertion and deletion mutant strains.
DP1322 Tn*5252* (*cat tet*)
SP1000 *str*-1 *fus* Tn*5252* (*cat*)
SP1004 *str*-1 *fus* Tn*5252* (Δ *cat*)
SP1019 *str*-1 *fus* Tn*5252* Em^r (Δ 3.22 kb *Cla*I fragment Δ *cat erm*)
SP1018 *str*-1 *fus* Tn*5252* Cm^r (Δ 23 kb *Hin*dIII fragment Δ *cat erm*)
SP1015 *str*-1 *fus* Tn*5252* Em^r (*cat erm*)
DP1333 Tn*5253* (Δ *cat tet*-3)
SP1014 Tn*5253* Em^r (Δ *cat tet*-3)
pVA891 Cm^r Em^r

chromosomal DNA strain: 16.8 kb, 4.55 kb, 2.06 kb, 1.92 kb, 1.09 kb, 1.03 kb, 0.85 kb; and to seven *Eco*RI fragments of the DP1322 control chromosomal DNA strain: 12.22 kb, 4.55 kb, 2.06 kb, 1.92 kb, 1.09 kb, 1.03 kb, 0.85 kb; and it reacts to the *Eco*RI termini fragments of the deleted region, 14 kb and 1.09 kb, of SP1018.

As expected, the ^{32}P-labeled pSS141 reacted to seven *Eco*RI fragments of SP1000 (Lane E, Figure 8): 16.8 kb, 4.55 kb, 2.06 kb, 1.92 kb, 1.09 kb, 1.03 kb, 0.85 kb; and the pSS141 probe reacted to seven *Eco*RI fragments of DP1322 (Lane F): 12.22 kb, 4.55 kb, 2.06 kb, 1.92 kb, 1.09 kb, 1.03 kb, 0.85 kb; and the same probe reacted to two *Eco*RI fragments of SP1018 (Lane A): 14 kb (the sum of a 11.82 kb and 2.2 kb *Hin*dIII fragment which replaced the deleted region) and the 1.09 kb (any fragment less than .85 kb did not appear in the original gel). Thus, the same probe did not react to any fragment within the deleted region of SP1018 indicating that the 23 kb *Hin*dIII fragment has been deleted from within Tn*5252*.

The pSS141 probe reacted weakly to many faint bands indicating a partial digest of the control DNA strains (Lane E and Lane F). In Figure 8, Lane D, it can be observed that the ^{32}P-labeled pSS141 reacted to two *Eco*RI fragments of the RxI chromosome, 3 kb and approximately 4.9 kb. The reaction of the pSS141 probe to the 3 kb *Eco*RI fragment was also observed in SP1018, DP1322 and SP1000. This indicated that

pSS141 has homology to the chromosome of pneumococcus and the reaction of the pSS141 probe to the 4.9 kb fragment might be due to the partial digest of the chromosomal DNA of Rx1.

The deletion 23 kb *Hin*dIII segment from within Tn*5252* can also be confirmed by using the *Hin*dIII restriction endonuclease digest of SP1018 chromosomal DNA. This can be observed when the ^{32}P-labeled pSS141 reacts to eight *Hin*dIII fragments of SP1000 chromosomal DNA: 6.8 kb, 3.8 kb, 3.59 kb, 3.33 kb, 2.7 kb, 1.65 kb, 1.34 kb, 0.98 kb; and it reacts to eight *Hin*dIII fragments of DP1322 chromosomal DNA: 6.8 kb, 3.8 kb, 3.59 kb, 3.33 kb, 2.7 kb, 1.65 kb, 1.34 kb, 0.98 kb; and the same probe pSS141 reacted to the right *Hin*dIII terminus fragment (3.59 kb) of the 23 kb *Hin*dIII deleted region of SP1018.

As expected, the ^{32}P-labeled pSS141 reacted to eight *Hin*dIII fragments of SP1000 chromosomal DNA (Figure 8, Lane L): 6.8 kb, 3.8 kb, 3.59 kb, 3.33 kb, 2.7 kb, 1.65 kb, 1.34 kb, 0.98 kb (any fragment less than 0.98 did not appear in the original gel) and it reacted to eight *Hin*dIII fragments of DP1322 chromosomal DNA (Lane M): 6.8 kb, 3.8 kb, 3.59 kb, 3.33 kb, 2.7 kb, 1.65 kb, 1.34 kb, 0.98 kb (very faint band); and the same probe pSS141 reacted to the 3.59 kb *Hin*dIII fragment of SP1018 (Lane H). This indicated that the 23 kb *Hin*dIII fragment has been deleted from within Tn*5252* in SP1018 deletion mutant strain.

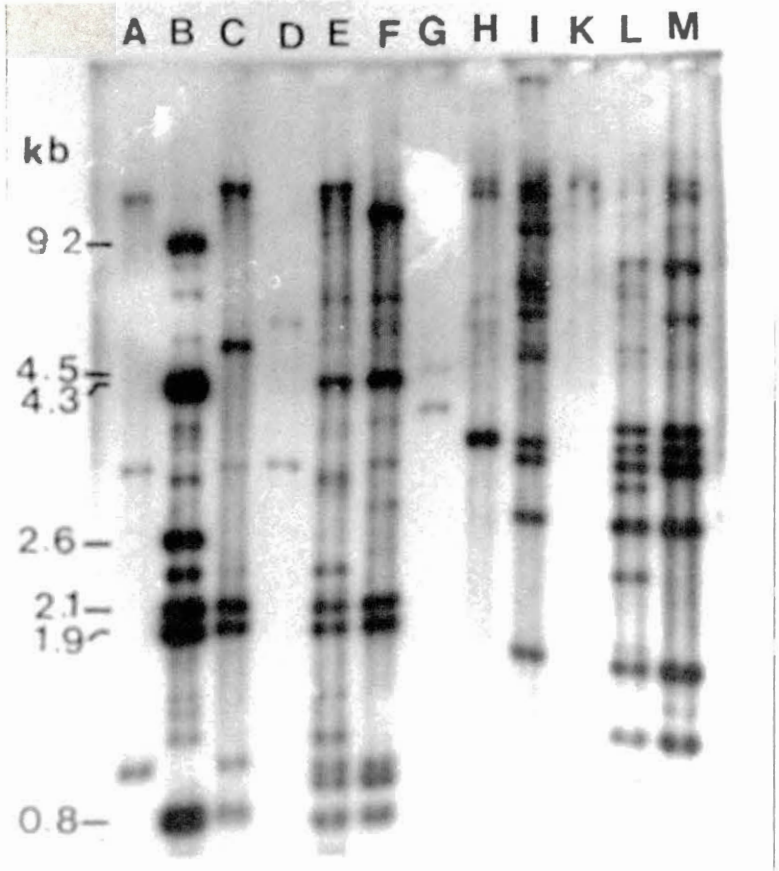

Figure 8. Autoradiogram of Southern Hybridization showing in vivo deletion of a 23 kb *Hin*dIII fragment from Tn*5252* (SP1018). The probe was ^{32}P-labeled pSS141. The probe reacted to the following *Eco*RI chromosomal DNA digest of (A) SP1018, (B) pSS141, (C) SP1019, (D) Rx1, (E) SP1000, (F) DP1322 and with *Hin*dIII chromosomal DNA digest of (H) SP1018, (I) SP1019,(K) Rx1, (L) SP1000, (M) DP1322

Furthermore, the probe reacted weakly to many extra *Hin*dIII bands of SP1000 in Lane L indicating a partial digest of the DNA. Moreover, the first two *Hin*dIII fragments in SP1018 (Lane H) were also observed in RxI, SP1000 and DP1333 (Lanes K, L and M, respectively) indicating that the probe pSS141 has homology to some regions in the chromosome.

In another experiment, a 1.34 kb *Hin*dIII fragment was used as a probe with the same chromosomal DNA of the SP1018 mutant strain, and the control strains, SP1000 and DP1322. The 1.34 kb *Hin*dIII fragment in Tn*5252* is present at the left end and within the 23 kb segment DNA (coordinate 5.5-6.9). In order to confirm 23 kb segment deletion, the ^{32}P-labeled 1.34 kb *Hin*dIII fragment should react to two *Eco*RI fragments, 4.55 kb and 1.03 kb, of the control strains, SP1000 and DP1322; and the same probe should react to one 1.34 kb *Hin*dIII fragment of control strain SP1000 and DP1322. On the other hand, with the deletion 23 kb segment of the SP1018 mutant strain neither the *Eco*RI nor *Hin*dIII fragment should hybridize with the probe.

As expected, the ^{32}P-labeled 1.34 kb *Hin*dIII fragment reacted to the 4.55 kb and 1.03 kb *Eco*RI fragments of the chromosomal DNA of the SP1000 and DP1322 control strains (Fig. 9, Lanes E and F); and the same probe reacted to the 1.34 kb *Hin*dIII fragment of the chromosomal DNA of the SP1000 and DP1322 control strains (Lanes L and M). On the

other hand, ^{32}P-labeled 1.34 kb *Hin*dIII fragment did not react to any *Eco*RI or *Hin*dIII fragment of SP1018 DNA (Fig. 9, Lanes A and H) indicating that the 23 kb *Hin*dIII region had been deleted from within Tn*5252* in the SP1018 mutant. This was additional evidence that the 23 kb *Hin*dIII fragment was deleted from within Tn*5252* in SP1018. Table 10 summarizes the reaction of the probes, pSS141 and the 1.34 kb *Hin*dIII fragment, to the SP1018 mutant strain.

Confirmation of the Deletion 3.22 kb of *Cla*I Fragment from within Tn*5252* in SP1019 by Southern Hybridization

It was difficult to confirm the 3.22 kb *Cla*I deletion fragment from within Tn*5252* of SP1019 by using pSS141 as a probe because the *Eco*RI and *Hin*dIII restriction endonuclease digests of SP1019 DNA (Fig. 8, Lanes C and I) were partial. Therefore, the evidence of the deletion of the 3.22 kb *Cla*I fragment was primarily obtained by using the 1.34 kb *Hin*dIII fragment as a probe.

If the 3.22 kb *Cla*I fragment is deleted from within Tn*5252* of the SP1019 mutant strain, the ^{32}P-labeled 1.34 kb *Hin*dIII fragment will react to the control strains, SP1000 and DP1322, and it will not react to any fragment of SP1019. This deletion can be confirmed when ^{32}P-labeled 1.34 kb *Hin*dIII fragment reacts to two *Eco*RI fragments, 4.55 kb and 1.03 kb, of the control strains SP1000 and DP1322 chromosomal DNA; and it will not react to any *Eco*RI fragment

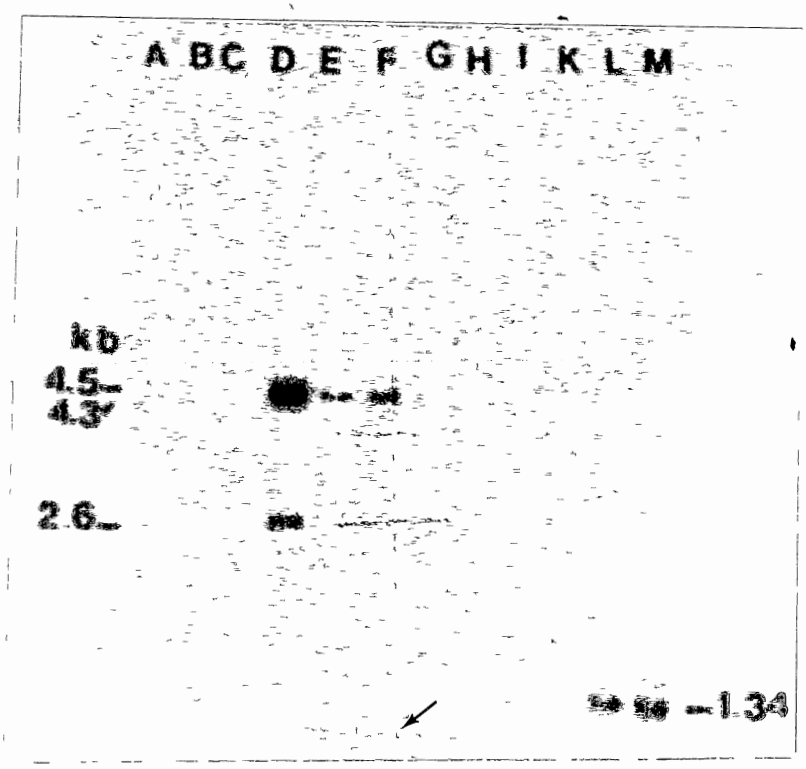

Figure 9. Autoradiogram of Southern hybridization showing in vivo deletion of a 23 kb *Hin*dIII fragment (SP1018) and the 3.22 kb *Cla*I fragment (SP1019) from Tn*5252*. The probe was ^{32}P-labeled 1.34 kb *Hin*dIII fragment. The probe reacted to the following *Eco*RI chromosomal DNA digest of (A) SP1018, (B) SP1019, (C) Rx1, (D) pSS141, (E) SP1000, (F) DP1322; and *Hin*dIII chromosomal DNA digest of (H) SP1018, (I) SP1019, (K) Rx1, (L) SP1000, (M) DP1322

TABLE 10

SUMMARY OF THE RESULTS OF HYBRIDIZATION BETWEEN THE TWO PROBES: pSS141 AND 1.34 kb *Hin*dIII FRAGMENT, AND CHROMOSOME OF THE SP1018 MUTANT STRAIN

	Sizes (kb) of reacting restriction fragments	
Strain	*Eco*RI	*Hin*dIII
<u>pSS141 as a probe</u>		
SP1018[b]	14, 1.09[a]	3.59
SP1000[c]	16.8, 4.55, 2.06 1.92, 1.09, 1.03, 0.85	6.8, 3.8 3.59, 3.33, 2.7, 1.65 1.34, 0.98[a]
DP1322	12.2, 4.55, 2.06 1.92, 1.09, 1.03, 0.85	6.8, 3.8 3.59, 3.33, 2.7, 1.65 1.34, 0.98
<u>1.34 kb *Hin*dIII as a probe</u>		
SP1018	n.d.	n.d.
SP1000	4.55, 1.03	1.34
DP1322	4.55, 1.03	1.34

a Any fragment less than 0.98 did not appear

b SP1000 contains Tn*5252*

C DP1322 contains Tn*5253*

n.d. not detected

of SP1019 chromosomal DNA.

As expected, 1.34 kb *Hin*dIII fragment reacted to two *Eco*RI fragments, 4.55 kb and 1.1 kb, of the control strains, SP1000 and DP1322 (Fig. 9, Lanes E, F). The same probe did not react to any *Eco*RI fragment of SP1019 (Lane B). This indicated that the 3.22 kb *Cla*I fragment was deleted from within Tn*5252* of SP1019.

Furthermore, the 3.22 kb *Cla*I deletion fragment can also be confirmed by using the *Hin*dIII restriction endonuclease digest of the SP1019 chromosomal DNA. The deletion will be observed when the ^{32}P-labeled 1.34 kb *Hin*dIII fragment reacts to the 1.34 kb *Hin*dIII fragment of the control strains, SP1000 and DP1322; and it will not react to any *Hin*dIII fragment of SP1019.

As expected, the ^{32}P-labeled 1.34 kb *Hin*dIII fragment reacted to the 1.34 kb *Hin*dIII fragment of the control strains SP1000 and DP1322 (Fig. 9, Lanes L, M); and the same probe did not react to any *Hin*dIII fragment of SP1019 chromosomal DNA (Lane I). This also confirmed that the 3.22 kb *Cla*I fragment was deleted from within Tn*5252* of SP1019. Table 11 summarizes the reaction of the 1.34 kb *Hin*dIII fragment with the DNA of the SP1019 mutant strain and the control strains.

TABLE 11

SUMMARY OF THE RESULTS OF HYBRIDIZATION BETWEEN THE 1.3 kb *Hin*dIII FRAGMENT PROBE AND CHROMOSOME OF THE SP1019 MUTANT STRAIN

	Sizes (kb) of reacting restriction fragments	
Strain	*Eco*RI	*Hin*dIII
SP1019	n.d.	n.d.
SP1000	4.55, 1.03	1.34
DP1322	4.55, 1.03	1.34

n.d. not detected.

Filter-Mating of the Mutant Strains SP1018, SP1019 and SP1015 with Various Recipients

To localize the essential regions of the Tn*5252* that control the conjugal transfer functions, SP1018, SP1019 and SP1015 were used as donors and *S. pyogenes* ATCC 35105 and *S. sanguis* ATCC 21547 as recipients in filter-mating experiments.

S. pyogenes and *S. sanguis* were optochin resistant and the mutant pneumococcal strains were optochin sensitive which facilitated the selection of the transconjugants in the filter-mating experiment. One major reason for using *S. pyogenes* and *S. sanguis* as recipients in filter-mating experiments was the chromosomal DNA of the two strains was expected to be heterologous to the chromosomal DNA of pneumococcus. In addition, *S. pyogenes* is incapable of natural transformation. These two properties excluded the chances of transformation and they allowed only for the interspecific conjugative transposition to occur during the filter-mating process. Moreover, to enhance the selection of the *S. pyogenes* and *S. sanguis* transconjugants, small volumes of the conjugation mixture were plated directly on the surface of the media with the appropriate combination of antibiotics in order to prevent the anaerobic pneumococcal donor mutants from growing.

In previous work, pVA891 was inserted in Tn*5253* by replacing 1.1 kb of the *tet* region and a GP42 strain was

created (42,43). This strain is Cm^r Tc^s Em^r Tra^+. In this work, GP42 was used as a control in filter-mating experiments after inserting a streptomycin chromosomal marker.

When the SP1018 mutant strain was used as a donor in filter-mating experiments and *S. pyogenes* as a recipient, the conjugative element in the SP1018 deletion strain was unable to transfer to *S. pyogenes*. In the control strain, the element transferred from GP42 to *S. pyogenes* indicating that the conjugal transfer property of SP1018 has been impaired.

To localize and narrow down the possible regions of the transfer-related gene(s), a SP1019 deletion mutant strain was created by deleting a smaller region, 3.22 kb *Cla*I fragment, within this 23 kb region (SP1018 Figure 6). Furthermore, SP1019 was used as a donor in filter-mating experiments and *S. pyogenes* as a recipient strain. Similarly, the element was unable to transfer from SP1019 to *S. pyogenes*. On the other hand, the element transfers from the GP42 to *S. pyogenes*. This indicated that the smaller region of the 3.22 kb *Cla*I fragment is also important for the conjugal transfer of the conjugative element Tn*5252*.

These results encouraged further localization of the transfer-related region by creating an insertional inactivation mutant in the same deleted region. Therefore, SP1015 was created by inserting pVA891 at the *Bgl*II site by

flanking homology as described previously. The SP1015 insertional inactivation mutant strain was used as a donor for the conjugative element in filter-mating experiments and *S. pyogenes* as a recipient strain. GP42 was used as a control donor strain. Similarly, the element was unable to transfer to *S. pyogenes* indicating that the transfer-related gene(s) have been impaired.

Conjugating the mutant strains with *S. pyogenes* was done twice in order to confirm the inability of the mutant strains to act as donors (Tables 12, 13). In the first group of filter-mating experiments (Table 12), the element transferred from GP42 to *S. pyogenes* at a frequency of 1.1 x 10^{-7} Em^r transconjugants per donor and 4.9 x 10^{-7} Cm^r transconjugants per donor indicating that the conditions of the filter-mating experiments were typical. These transconjugants were replica plated and observed to be Em^r Cm^r Opt^r Str^s.

In the second group of filter-mating experiments (Table 15), the element transferred from the GP42 to *S. pyogenes* at a frequency of 5.2 x 10^{-7} Em^r Cm^r transconjugants per donor indicating that the conditions of the filter-mating experiment were typical. These transconjugants were also replica plated and were observed to be Em^r Cm^r Opt^r Str^s.

To further confirm the inability of the mutant strains to act as donors for the conjugative element, *S. sanguis* was used as a recipient strain, GP42 as a control donor strain,

and SP1015, SP1019 and SP1018 mutant strains as donors in filter-mating experiments. Again, the element Tn*5252* was incapable of transferring from the three mutant strains SP1015, SP1018 and SP1019 to *S. sanguis* indicating that the conjugal transfer of Tn*5252* has been impaired. At the same time, the element in the control strain GP42 transfers to *S. sanguis* at a frequency of 6×10^{-9} Cm^r transconjugants per donor indicating that the filter-mating conditions were typical (Table 14). These transconjugants were replica plated and were observed to be Em^r Cm^r Opt^r Str^s. Thus, the selection of Em^r transconjugants was not successful indicating that the selection of erythromycin transconjugants is not efficient in *S. sanguis*.

TABLE 12

THE FIRST CONJUGATION EXPERIMENT BY FILTER-MATING USING THE MUTANT STRAINS SP1015, SP1018 AND SP1019 AS DONORS AND *S. pyogenes* AS RECIPIENT

	Viable count (per ml)			
Donor	Donor	Recipient[a]	Selection	Transcojugants per donor
GP42	1.4×10^9	5.3×10^9	Opt 20[b]/Em 5[c]	1.1×10^{-7}
			Opt 20/Cm 5[d]	4.9×10^{-7}
SP1015	1.1×10^9	12×10^9	Opt 20/Em 5	$<2.2 \times 10^{-9}$
			Opt 20/Cm 5	n.t
SP1018	1.2×10^9	9.8×10^9	Opt 20/Cm 5	$<2 \times 10^{-9}$
SP1019	1.4×10^9	12.4×10^9	Opt 20/Em 5	$<1.7 \times 10^{-9}$

a The recipient strain was always *S. pyogenes*.

b 20 μg of Optochin (ethylhydrocupriene) per ml.

C 5 μg of erythromycin per ml.

d 5 μg of chloramphenicol per ml.

n.t. not tested.

TABLE 13

THE SECOND CONJUGATION EXPERIMENT BY FILTER-MATING USING MUTANT STRAINS SP1015, SP1018 AND SP1019 AS DONORS AND *S. pyogenes* AS RECIPIENT

Donor	Viable count (per ml)		Selection	Transconjugants per donor
	Donor	Recipient[a]		
GP42	3.5×10^9	20×10^9	Opt 20[b]/Em 5[c]	5.2×10^{-7}
			Opt 20/Cm 5[d]	5.2×10^{-7}
SP1015	6×10^9	24×10^9	Opt 20/Em 5	$<0.3 \times 10^{-9}$
			Opt 20/Cm 5	$<0.3 \times 10^{-9}$
SP1018	7.1×10^9	21.2×10^9	Opt 20/Cm 5	$<0.1 \times 10^{-9}$
SP1019	3.5×10^9	20.1×10^9	Opt 20/Em 5	$<0.2 \times 10^{-9}$

a The recipient strain was always *S. pyogenes*.

b 20 μg of optochin (ethylhydrocupriene) per ml.

C 5 μg of erythromycin per ml.

d 5 μg of chloramphenicol per ml.

TABLE 14

CONJUGATION BY FILTER-MATING USING THE MUTANT STRAINS SP1015, SP1018 AND SP1019 AS DONORS AND *S. sanguis* AS RECIPIENT

Donor	Viable count (per ml)		Selection	Transconjugants per donor
	Donor	Recipient[a]		
GP42	1.4×10^9	7×10^{10}	Opt 20[b]/Em 5[c]	$<1.4 \times 10^{-9}$
			Opt 20/Cm 5[d]	6×10^{-9}
SP1015	2.7×10^9	9×10^{10}	Opt 20/Em 5	$<0.7 \times 10^{-9}$
			Opt 20/Cm 5	$<0.7 \times 10^{-9}$
SP1018	1.6×10^9	8×10^{10}	Opt 20/Cm 5	$<0.6 \times 10^{-9}$
SP1019	1.8×10^9	6×10^{10}	Opt 20/Em 5	$<0.5 \times 10^{-9}$

a The recipient strain was always *S. sanguis*.

b 20 μg of optochin (ethylhydrocupriene) per ml.

C 5 μg of erythromycin per ml.

d 5 μg of chloramphenicol per ml.

CHAPTER IV

DISCUSSION

Gene rearrangements have always been observed in the eukaryotes' and prokaryotes' genome in the past decades. These rearrangements play a functional role in controlling gene expression and cell differentiation (9). A typical example of the evolution and development of gene rearrangement was the role of *tra* genes in conjugative plasmids and conjugative transposons. Thus, the existence of the conjugative plasmids and conjugative transposons focused attention on the possibility of a controlled DNA rearrangement through the DNA transfer mechanism.

One might ask; what is the selection pressure responsible for the origin and the maintenance of the *tra* genes? Novick (25) has suggested that the *tra* function is very important for the evolution and the survival of the conjugative plasmids. In fact, one third of the F genome plasmid in *E. coli* is devoted to the *tra* function. This includes at least 22 genes of the *tra* family (16). Clewell (8) has suggested that *tra* genes of the conjugative transposons might initially be acquired through an association with conjugative plasmids.

In recent years, DNA sequence and protein analysis revealed that the *tra* proteins of Tn*1545* demonstrated some homology to the Xis and Int proteins of the lambda phage. This was also emphasized when the *tra* proteins of the pSAM2 conjugative plasmid of *Streptomyces ambofacience* displayed local homology with the Xis and Int proteins of the lambda phage (28). This discovery indicated that the origin of the *tra* genes of the conjugative plasmids, conjugative transposons, and lambda phages came perhaps from related ancestors.

Although the current hypothesis for the mobility mechanism of the conjugative transposons is far from being understood, several investigators (33,41) suggested that after the excision of the element, a non-replicative circular intermediate of the element undergoes intracellular or intercellular transposition to a new site. The excision and the insertion mechanism are the major keys in the conjugative transposition mechanism. Furthermore, the excisinase and integrase proteins have been identified and isolated in Tn*916* and Tn*1545*. Similarly, the circular intermediate has been captured and isolated in Tn*916* and Tn*1545* (41).

The complex function of the *tra* genes for the mobilization of the conjugative element has suggested that major segments of the conjugative element should be involved in the conjugal transfer mechanism. Sanger et al. (31)

suggested that major segments of Tn*916* were devoted to conjugative function. This was concluded when Tn*5* was inserted at different locations along the conjugative element and most of the mutants exhibited a Tra$^-$ phenotype.

In this work, the location of one region of the potential transfer-related gene(s) of Tn*5252* was investigated and *tra*$^-$ mutant strains have been successfully created.

The Genetic and Physical Analysis of pSS141

On the basis of genetic analysis, cloning of the 21 kb *KpnI* fragment from the left side of Tn*5253* (excluding the *cat* region) has led to the construction of pSS141. The cloning strategy of inserting pVA891 in Tn*5253* by flanking homology is more efficient than inserting pVA891 by insertion duplication of the directed DNA. This successful strategy is consistent with the work of Pozzi et al. (27).

On the basis of physical restriction analysis of pSS141, it appears that pVA891 is inserted at a *Bgl*II site in Tn*5253* of SP1014 (coordinate 5.5) by flanking homology. The physical measurements of the passenger DNA confirmed this. Similarly, SP1015 is expected to have pVA891 inserted in the same *Bgl*II site upon using SP1014 DNA as a donor DNA in transforming SP1000 competent cells. Thus, replica plating for SP1015 indicated that SP1015 is Em^r Cm^r Str^r.

Physical Analysis of the Mutant Strains

The in vivo deletion mutagenesis was conducted by using the fusion fragment as a directing DNA to integrate into the conjugative element, Tn*5252*. The deletion of the 23 kb *Hin*dIII segment (including the *cat* region) and the 3.22 kb *Cla*I fragment has been described in Chapter III. The transformants obtained from the deletion of the 23 kb *Hin*dIII segment (50 Cm^r transformants per ml, Table 8) were higher than the transformants obtained from deleting the 3.22 kb *Cla*I fragment (5 Em^r transformants per ml, Table 9).

This difference in transformation frequency can be explained by the fact that the ligated molecule that was used to delete the 3.22 kb *Cla*I fragment was around 23 kb; the possibility of the entry of a long single-stranded DNA into the competent cell without interruption was low (14,30) compared to a 6.8 kb fragment, which was used to delete a 23 kb *Hin*dIII fragment in SP1018 (14,30).

Further, the deletions from within Tn*5252* in SP1018 and SP1019 were confirmed by Southern hybridization. In the SP1018 mutant strain, Southern hybridization analysis indicated that the ^{32}P-labeled probe pSS141 reacted to the left termini and the right termini of the regions flanking the deletion fragment and did not react to any fragment within the deleted region (Table 10, Fig. 8). This suggested that 23 kb *Hin*dIII segment had actually been

deleted from Tn*5252*. Similarly, in SP1019, Southern hybridization analysis revealed that the ^{32}P-labeled 1.34 kb *Hin*dIII fragment did not react to any *Eco*RI or *Hin*dIII fragments within the deleted region, confirming the deletion of the 3.22 kb *Cla*I fragment (Table 11, Fig. 9).

Conjugal Transfer Analysis of the Mutant Strains SP1018, SP1019 and SP1015

After the construction of the mutant strains SP1019 (Tn*5252* ⌂ 3.22 kb *Cla*I fragment), SP1018 (Tn*5252* ⌂ 23 kb *Hin*dIII segment), and SP1015 (Em^r Tn*5252 cat erm*), conjugal transfer studies were conducted to investigate the ability of the three mutant strains to act as donors in filter-mating experiments.

Filter-mating studies indicated that the three mutant strains were unable to act as donors for the conjugative element to *S. pyogenes* (two separate experiments) and to *S. sanguis*. In these filter-mating experiments GP42 was used as a control donor strain to confirm the typical condition of the conjugation experiments. This result suggested that deleting the 23 kb *Hin*dIII segment, the 3.22 *Cla*I fragment, and inserting pVA891 at the *Bgl*II site of Tn*5252* affected the conjugal transfer mechanism of the conjugative element. It should be noted that the three mutant strains overlapped in one region and our results suggested that the 3.39 kb *Xba*I fragment region (coordinates 4.5-8) is important ın the

conjugal transfer of the element Tn*5252*.

Although many studies (5,7,8,20) have shown that transfer-related genes are most likely located near the extremities of the conjugative elements, it would be interesting to investigate the functional role of additional segments along the conjugative element Tn*5252*. Therefore, in additional experiments to localize more regions important for the conjugal transfer, we inserted pVA891 at different locations of the 8.11 kb *Bgl*II fragment (coordinates 19.5-27.4) of the conjugative element Tn*5252*. This work was done by electroeluting the 8.11 kb *Bgl*II fragment from pSS141, digesting the fragment with *Sau*3AI restriction endonuclease, and ligating the molecules to pVA891 which was digested with *Bam*HI restriction endonuclease. Then the ligated molecules were added to SP1000 (Tn*5252*) competent cells and eight transformants were isolated. Subsequently, Southern hybridization analysis was conducted to localize the pVA891 insertion in the conjugative element. Unfortunately, because of the constraints of time, this work was not completed. It would be worthwhile to investigate the conjugal transfer properties for these mutants and localize more regions that are important for the *tra* function of the conjugative element Tn*5252*.

Conclusion and Recommendations

The data are sufficient to assume that either the

insertion of pVA891 at the *Bgl*II site, or the deletion of the 3.22 *Cla*I or the 23 kb *Hin*dIII segment affected the conjugative transposition of Tn*5252*. Thus, further investigation of the functional properties of the *tra*⁻ mutant strains is warranted.

One way to study these properties is by conducting an in vivo trans-complementation assay. This should be conducted by introducing the mutated region, the 3.39 kb *Xba*I fragment, on a recombinant plasmid, and subsequently finding out whether the conjugal transfer of the Tn*5252* is restored upon introducing the recombinant plasmid into mutant strains.

Therefore, the *Xba*I fragment passenger DNA of Tn*5252* will be isolated from pVJ438 and pLS1 recombinant plasmid will be digested with *Eco*RI restriction endonuclease. The cohesive ends of the *Xba*I fragment and *Eco*RI cohesive ends of pLS1 will be removed by nuclease S1 treatment which will remove the single-stranded tails from the DNA producing blunt ends. Furthermore, blunt ends of the *Xba*I fragment will be ligated to the blunt ends of *Eco*RI-pLS1 recombinant plasmid (16). The pLS1 plasmid is 4.3 kb and carries tetracycline resistance that can be expressed in *S. pneumoniae* (2,40) and this plasmid can replicate autonomously in *S. pneumoniae*. Afterwards, the blunt end ligated molecule will be added to RxI competent cells and the transformants will be selected on media containing

tetracycline. Restriction enzyme analysis will be followed to identify the recombinant plasmids that contain the *Xba*I passenger DNA.

In further work, the recombinant plasmid carrying the *Xba*I passenger DNA fragment of Tn*5252* will be introduced to the *tra*⁻ mutant strains and the restoration of the conjugal transfer mechanism of Tn*5252* will be investigated.

If the in vivo trans-complementation assay restores the conjugal transfer mechanism of Tn*5252*, this would indicate that the 3.39 kb *Xba*I fragment of Tn*5252* produces diffusible proteins that can complement the mutant strains and make them transfer proficient.

Furthermore, protein profile analysis should be conducted to identify the functional properties of these diffusible proteins. On the other hand, if the trans-complementation assay does not work, cis-complementation assay should be conducted and the conjugative trasnposition of the mutant strains should be investigated. This experiment will investigate the possibility of whether the proteins which are involved in conjugative transposition are cis-acting proteins.

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