# STRUCTURAL AND FUNCTIONAL CHARACTERIZATION OF A COMPOSITE CONJUGATIVE TRANSPOSON IN *STREPTOCOCCUS PNEUMONIAE*

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ABSTRACT

Extensive homology between the _tet_ determinant and the flanking regions among most of the streptococcal conjugative transposons suggested that this novel class of mobile elements could have arisen by the insertion of heterologous elements into a basic _tet_ unit, as represented by the smallest known conjugative transposon, Tn_916_. However, the homology between Tn_916_ and the 65 kb Tn_5253_ was limited only to the _tet_ region and did not extend to any other region. Moreover, an 18 kb segment carrying the _tet_ determinant that is totally internal to Tn_5253_ was found to be an independent conjugative transposon, Tn_5251_. We sought to determine the role of the sequences beyond Tn_5251_ within Tn_5253_ in transposition. To this aim, the segment of DNA containing Tn_5251_ was deleted from within Tn_5253_ and the deletion mutant strain was used in filter-matings to determine whether the altered element retained its transposition properties.

The sequences beyond Tn_5251_ within Tn_5253_, designated Tn_5252_, transferred by filter-mating to _Streptococcus pneumoniae_ and _Enterococcus faecalis_ recipient cells at a frequency comparable to that of the parental element. Differences in the structural and functional properties between Tn_5251_ and Tn_5252_ seemed to

suggest that these belonged to two distinct classes of mobile elements.

Moreover, Tn5253, originally detected in a clinical isolate of S. pneumoniae, is indeed a composite element comprised of two independent conjugative transposons, Tn5252 and Tn5251. These results can be extrapolated to the conclusion that the prototype for this type of element is perhaps a Tn5252-like transposon and not the tet carrying Tn916-like element.

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TABLE OF CONTENTS

Chapter Page

I. INTRODUCTION . . . . . . . . . . . . . . . . . . . 1

Antibiotic Resistance in Pneumococcus . . 1
Features of Bacterial Conjugation . . . . 2
Streptococcal Conjugative Transposons . . 3

II. MATERIALS AND METHODS. . . . . . . . . . . . . . 11

Bacterial Strains and Plasmids. . . . . . 11
Enzymes and Chemicals . . . . . . . . . . 15
Growth and Storage of Bacterial Strains . 15
Preparation of Chromosomal DNA. . . . . . 18
Preparation of Plasmid DNA. . . . . . . . 19
Transformation. . . . . . . . . . . . . . 20
Agarose Gel Electrophoresis . . . . . . . 20
DNA-DNA Hybridization . . . . . . . . . . 21
Conjugation . . . . . . . . . . . . . . . 21

III. RESULTS. . . . . . . . . . . . . . . . . . . . 23

In Vivo Deletion of Tn5251 from Tn5253. . 25
Generation of pVJ403Δtet. . . . . . 25
In Vivo Deletion of Tn5251 from Tn5253. . . . . . . . . . . 27
Genetic Analysis of Tc$^s$ Isolates by Transformation . . . . . . . . . . 30
Physical Analysis of Tc$^s$ Isolates by Southern Hybridization . . . . . . 31
Conjugative Transfer Properties of Tn5253ΔTn5251. . . . . . . . . . . . . 34
Intraspecific Conjugative Transfer of Tn5253ΔTn5251. . . . . . . . . 34
Target Preference of Tn5252 in Pneumococcus . . . . . . . . . . . 38
Interspecific Conjugative Transfer of Tn5252. . . . . . . . . . . . . 41
Genetic Analysis of the UV202 Transconjugants by transformation. 44
Physical Analysis of the UV202 Transconjugants by Southern Hybridization. . . . . . . . . . 46

IV. DISCUSSION . . . . . . . . . . . . . . . . . . . 61

LITERATURE CITED. . . . . . . . . . . . . . . . . . . 72

## LIST OF TABLES

Table Page

1. Pneumococcal Strains. . . . . . . . . . . . . . 12

2. E. coli Strains and Cloning Vectors . . . . . 14

3. Selective Antibiotic Concentrations . . . . . 17

4. Transformation of DP1333 with Crude Lysates of Ten $Tc^s$ Isolates . . . . . . . . . . . . 32

5. Transfer of Tn5253ΔTn5251 to DP1002 by Filter-mating . . . . . . . . . . . . . . . 37

6. Conjugation by Filter-mating from SP1002 Donors . . . . . . . . . . . . . . . 39

7. Transfer of Tn5252 to E. faecalis UV202 by Filter-mating. . . . . . . . . . . . . . 43

8. Analysis of the UV202 Transconjugants by Transformation . . . . . . . . . . . . . 45

9. Summary of Results of Hybridization Between pVJ407 and Two UV202 Transconjugants. . . . 57

10. Summary of Results of Hybridization Between pVJ414 and Two UV202 Transconjugants. . . . 59

LIST OF FIGURES


| Figure | | Page |
|---|---|---|
| 1. | Physical Map of Tn5253 . . . . . . . . . . . . . . | 7 |
| 2. | Restriction Endonuclease Map of pVJ403 and the Deletion Derivative, pVJ403Δ tet . . . . . | 26 |
| 3. | Strategy for the In Vivo Deletion of Tn5251 from Tn5253. . . . . . . . . . . . . . . . . . | 29 |
| 4. | Autoradiogram of Southern Hybridization Showing the In Vivo Deletion of Tn5251 from Tn5253 . . | 33 |
| 5. | Autoradiogram of Southern Hybridization Showing Target Preference of Tn5252 in Pneumococcus. . | 40 |
| 6. | Hybridization Between the Tn5253 Target in Rx1 and the Chromosomal DNA of Various Streptococcal Species. . . . . . . . . . . . . | 48 |
| 7. | Analysis of the central region of Tn5252 and Tn5253 in *E. faecalis* UV202 transconjugants. . . | 50 |
| 8. | Analysis of the Right Junction Region of *E. faecalis* UV202 transconjugants . . . . . . . . | 53 |
| 9. | Analysis of the Right Junction Region of two *E. faecalis* UV202 transconjugants . . . . . . . . | 56 |
| 10. | Analysis of the Left Junction Region of two *E. faecalis* UV202 transconjugants . . . . . . . . | 58 |

CHAPTER I

INTRODUCTION

**Antibiotic resistance in pneumococcus.** Antibiotic resistance in bacteria has traditionally been associated with plasmids. As with most bacterial species, resistance plasmids are common in streptococci with the interesting exception of Streptococcus pneumoniae (pneumococcus). However, when many multiply resistant strains of pneumococcus began to appear in the late 1970's (21), attempts to identify resistance plasmids in these isolates were unsuccessful (6,31,36,44). Further studies revealed the chromosomal location of the resistance determinants (11,13,16,34) in these strains. Many of the chromosomal resistance determinants were able to transfer by a DNase I resistant process resembling classical conjugation both within and between different species of streptococci (3,7,12,13,18,35). Properties of some of the conjugative elements have shown that these were also transposable (11,13,19). Hence, the term "conjugative transposon" has been applied to them. The horizontal spread of antibiotic resistance among streptococci appears to be chiefly due to these conjugative transposons (9). As streptococci represent the fifth leading cause of mortality in the elderly and

very young, continued study of these conjugative elements is of both medical and biological concern.

**Features of bacterial conjugation.** Conjugation in bacteria is not a unique phenomenon. Much is known about conjugation mediated by the fertility factor (F factor) in E. coli (42), first described thirty years ago. The F factor resides on a plasmid replicon, the F plasmid. Insertion sequences (specifically, IS2 and IS3) located both on the E. coli chromosome and on the F plasmid allow integration of the F factor into the host chromosome by a homology dependent recombination process. The integration of the F factor into the E. coli chromosome is rec dependent. When the F factor integrates into the chromosome, conjugative transfer results in mobilization of the flanking chromosomal DNA of the donor into the recipient cells. The DNA transfer is unidirectional through the F-pilus and mating occurs in broth. The F factor can transfer only between E. coli and very closely related species.

Conjugation mediated by streptococcal conjugative transposons differs in many ways from E. coli F factor conjugation. These streptococcal conjugative elements lack autonomous replication functions and must integrate into a chromosomal or a plasmid replicon for maintenance and propagation (13). At least for Tn916, integration is not dependent upon homology and host recombination systems, as expected of a transposable element (28). Co-

transfer of chromosomal markers has not been reported during conjugation mediated by these elements. Intimate cell-to-cell contact is required for conjugation such as that provided during mating on nitrocellulose filters (13,35). In contrast to the F factor in E. coli, these elements are also capable of interspecific conjugal transfer (4,38).

**Streptococcal conjugative transposons.** Conjugative transposons have been isolated from a variety of streptococcal species. These elements range in size from 16 kb to over 60 kb. While many carry multiple drug resistance determinants, all of the streptococcal conjugative transposons studied thus far contain a tetracycline (tet) resistance determinant (3). Based on transformation analysis, Smith, et al. observed significant homology among the tet determinants carried by a variety of streptococcal conjugative transposons (38). The distribution of a homologous tet determinant among these streptococcal conjugative elements raised questions concerning the evolution of such elements. The possibility has been raised that a tet containing element similar to Tn916 (described below) represented a prototype conjugative transposon serving as a receptacle for the accumulation of other heterologous elements resulting in the increase in size and multiple antibiotic resistance (9,16).

The first and possibly the best characterized

streptococcal conjugative transposon is Tn916 from Enterococcus faecalis (formerly Streptococcus faecalis) DS16 (13). This element is 16 kb and confers resistance to tetracycline ($Tc^r$) (32). This conjugative transposon transfers to streptococcal recipient cells at a frequency of $10^{-5}$ transconjugants per donor (13). Tn916 is capable of both intracellular and intercellular transfer and inserts into randomly chosen sites on the recipient chromosome (14). When DNA containing this element was cloned into an E. coli plasmid vector, Tn916 precisely excised in the absence of selective pressure. DNA sequence analysis of the termini of Tn916 has revealed short, direct and inverted imperfect repeats. Additional sequence studies of the target sites have revealed a short consensus sequence at the target with no duplication of the target sequences (8).

S. pneumoniae BM4200 originally isolated by Courvalin, et al. carries resistances to erythromycin ($Em^r$), kanamycin ($Km^r$), tetracycline ($Tc^r$), and chloramphenicol ($Cm^r$) (12). Based on transformation criteria, Smith, et al. (38) estimated the length of the conjugative BM4200 element to be more than 60 kb. However, the conjugative transposon, Tn1545, carrying erythromycin, kanamycin, and tetracycline resistance determinants and derived from this strain, was only 25 kb (12). This discrepancy in size and the absence of a chloramphenicol determinant on this mobile element still

remains to be accounted for.

Tn3701, carried by S. pyogenes A454, is greater than 50 kb and encodes resistance to erythromycin ($Em^r$) and tetracycline ($Tc^r$) (23). Through DNA-DNA hybridization analysis, Le Bouguenec, et al. identified a 19 kb segment of DNA within Tn3701 with significant homology to Tn916 (24). This segment of DNA, designated Tn3703, carries both the tet and erm resistance determinants.

S. agalactiae B109 contains a large conjugative transposon termed Tn3951. This element is 67 kb and contains chloramphenicol ($Cm^r$), erythromycin ($Em^r$), and tetracycline ($Tc^r$) resistance determinants (20). Following conjugative transfer to pneumococcus, this element integrates within a preferred target site in the pneumococcal chromosome (30).

Studies in this laboratory have focused on Tn5253, formerly termed Ω(cat tet)BM6001, from S. pneumoniae BM6001 which carries resistances to chloramphenicol ($Cm^r$) and tetracycline ($Tc^r$) (39) and transfers to pneumococcal recipient cells at a frequency of $10^{-7}$ to $10^{-6}$ transconjugants per donor (37). Using directed insertional mutagenesis, a series of recombinant plasmids containing passenger DNA from this element has been constructed in E. coli (40). Physical analysis of the passenger DNA from these clones has produced a reliable and consistent restriction endonuclease map of Tn5253 (Figure 1, 39). This 65.5 kb conjugative element

integrates within a preferred target site in the pneumococcal chromosome and this appears to be the same target site into which Tn3951 integrates within the pneumococcal chromosome (39).

With this library of clones and physical map of Tn5253 one can begin to approach several important questions which could not have been addressed before. Focusing on Tn5253 as a model, one interesting and immediate question that we can now address concerns the evolution of these streptococcal conjugative elements. Is it true that all of these conjugative elements are derived from a common ancestor? Although, based on phenotypic characteristics, these elements appear to be of common ancestry, more recent observations seem to conflict with this hypothesis. Consider the following observations.

First, if a Tn916-like element is the prototype for all streptococcal conjugative transposons into which other sequences have inserted resulting in an increase in size, one expects the termini of such elements to be maintained and retain some degree of homology to Tn916. To address this, Le Bouguenec, et al. (24) used Tn916 and five different fragments of Tn3701 as probes in Southern hybridization to determine the extent of homology among several different streptococcal conjugative transposons. They found that the larger elements (Tn5253, Tn3701, and Tn3951) all contain a central region surrounding the tet

ΩBM6001 ELEMENT (Tn 5253)

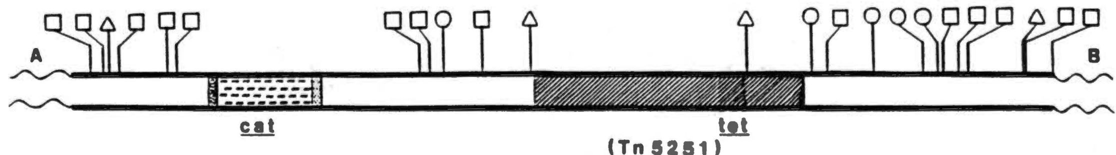

Figure 1. Physical map of Tn5253. ═══ , Tn5253 DNA; ≈≈ , pneumococcal chromosomal DNA; A and B refer to the left and right termini of Tn5253 respectively; [dashed box], cat region; [stippled box], direct repeats flanking the cat determinant; [hatched box], Tn5251 DNA; [square], XbaI; [triangle], KpnI; [circle], BamHI.

determinant with significant homology to Tn916. The smaller Tn1545 element was also homologous to Tn916 and the homology extended to the termini. Indeed, DNA sequencing studies have shown that at least 250 bases of the termini of Tn916 and Tn1545 are perfectly homologous (5,8).

Similarly, when we used Tn916 as a probe in hybridization with several different recombinant plasmids containing passenger DNA derived from the various regions of the Tn5253 element, the probe was seen to hybridize to only those plasmid clones derived from the tet region of Tn5253. All homology to Tn916 was localized in this central tet region. As the sizes of the junction regions of this element in pneumococcus are well known (39) and the probe failed to react with DNA fragments of such sizes (1), we were able to infer that Tn916 probably did not carry any homology to the termini of Tn5253.

A second observation was provided by the findings of Kilic, a colleague working in this laboratory. When cloned onto an E. coli plasmid vector, the Tn916-like sequences from the central region of Tn5253 spontaneously excised in the absence of tetracycline selection. When introduced into wild type pneumococcus by transformation, Kilic observed that the Tn916-like sequences transposed as a unit from this recombinant plasmid into the pneumococcal chromosome at random sites giving rise to $Tc^r$ transformants while the flanking sequences and the

vector portion of this plasmid were lost. In addition, this Tn916-like transposon transferred from pneumococcal donor cells by conjugation to pneumococcal recipient cells at a frequency of about $10^{-5}$ per donor during filter-mating. This 18 kb conjugative transposon, homologous to Tn916, was designated Tn5251 (1).

Together, these two lines of evidence strongly argue against the Tn916 prototype model. While it is likely that the smaller elements such as Tn5251 and Tn1545 are derivatives of a Tn916-like progenitor as their homology to Tn916 extends throughout the elements and to the termini, the observed structural and functional properties of the larger conjugative elements conflict with this model. Is it possible that these larger conjugative elements containing a homologous tet determinant are the products of insertion of a Tn916-like element into another element that is not structurally related to the former class of transposons?

Do the sequences beyond Tn5251 in Tn5253 possess conjugative properties? If not, how does the smaller independent transposon recognize terminal sequences 20 to 30 kb away on either side to initiate transfer? Yet, how does it no longer recognize its own ends as it mobilizes Tn5253? In an attempt to address these questions, one must first observe the properties of Tn5253 without the Tn5251 sequences.

In this work, we approached this problem and present

evidence to show that the sequences beyond Tn5251 within Tn5253 do constitute a transposon capable of intraspecific and interspecific transfer and that the smaller Tn5251 transposon does not play a mechanistic role when present as a part of the larger element. In addition, the results obtained in this work showed that these two conjugative elements probably belong to two different classes of transposons.

## CHAPTER II

## MATERIALS AND METHODS

**Bacterial strains and plasmids.** The non-encapsulated laboratory strain equivalent to wild type Streptococcus pneumoniae used in these studies is Rx1 (34). Other pneumococcal strains (Table 1) are derivatives of Rx1. DP1333 ($Tc^{s}$ $Cm^{s}$ $tra^{+}$) is DP1322 which acquired the tet-3 point mutation conferring sensitivity and spontaneously lost cat. DP1617 has multiple chromosomal markers and its chromosomal DNA was used as a reference in transformation procedures. Escherichia coli DH5α (Table 2), a recombination deficient strain, was used primarily for generation and preparation of recombinant plasmids. The recombination deficient strain of Enterococcus faecalis, UV202 (43), was used in some conjugation experiments. E. faecalis UV202 contains two chromosomal point mutations conferring resistance to rifampicin ($Rif^{r}$) and fusidic acid ($Fus^{r}$). Cloning vectors used in these studies are listed in Table 2. The plasmid, pVA891 confers resistance to erythromycin in streptococci and in E. coli but can autonomously replicate only in E. coli. In addition, chloramphenicol resistance of pVA891 is expressed in E. coli but not in streptococci.

TABLE 1

PNEUMOCOCCAL STRAINS

| Strain | Genotype | Reference |
| --- | --- | --- |
| Rx1 | hex | Shoemaker, et al. 1974. (33) |
| DP1002 | nov-1 | Guild, et al. 1976. (15) |
| DP1004 | str-1 | Guild, et al. 1976. (15) |
| DP1322 | Tn5253 (cat tet) | Shoemaker, et al. 1979. (34) |
| DP1324 | str-1 Tn5253 (cat tet) | Shoemaker, et al. 1980. (35) |
| DP1333 | Tn5253 ( cat tet-3) | Smith, et al. 1981. (38) |
| DP1617 | $hex^+$ str-1 ery-2 nov-1 fus sulf-d stg | Smith, et al. 1981. (38) |
| SP1000 | str-1 Tn5252 (cat) | This study |
| SP1001 | str-1 Tn5252 $Em^r$ (cat erm) | This study |
| SP1002 | nov-1 Tn5252 $Em^r$ (cat erm) | This study |

nov-1 Chromosomal point mutation conferring resistance to novobiocin.

str-1 Chromosomal point mutation conferring resistance to streptomycin.

cat chloramphenicol acetyl transferase.

tet tetracycline resistance of the type M.

ery-2 Chromosomal point mutation conferring resistance to erythromycin.

fus Chromosomal point mutation conferring resistance

to fusidic acid.

sulf-d Chromosomal point mutation conferring resistance to sulfanilamide.

stg Chromosomal point mutation conferring resistance to streptolidigin.

Em erythromycin.

TABLE 2

*E. COLI* STRAINS AND CLONING VECTORS

| Strain | Relevant Genotype or Phenotype | Source or Reference |
| --- | --- | --- |
| DH5α | $F^-$ recA1 $lacZ^+$ | Bethesda Research Laboratories |
| pACYC184 | 4.2 kb conferring $Cm^r$ and $Tc^r$ | |
| pBluescript (KS+ and SK+) | 2.7 kb, $lacZ^+$, $Amp^r$ | Stratagene |
| pUC8 and pUC19 | 2.7 kb, $lacZ^+$, $Amp^r$ | |
| pVA891 | 5.9 kb, $Cm^r$, $Em^r$ | Macrina, et al. 1983. (25) and Vijayakumar, et al. 1986. (40) |

Cm chloramphenicol

Tc tetracycline

Amp ampicillin

Em erythromycin

**Enzymes and chemicals.** Restriction endonucleases, T4 DNA ligase, DNA molecular weight standards and *E. coli* DNA Polymerase I were purchased from Bethesda Research Laboratories, Promega Biotec, or International Biotechnologies, Inc. DNase I, RNase I, egg white lysozyme (grade 1), and bovine serum albumen (fraction V) were purchased from Sigma. Media and agar for bacterial growth were purchased from Difco, except for casein hydrolysate which was purchased from United States Biochemical Company (USBC). Agarose from Fisher or International Biotechnologies, Inc. was used for horizontal gel electrophoresis. Ultrapure agarose from Bio-Rad Laboratories was used when isolating defined DNA restriction fragments by electroelution for subcloning. [$\alpha$-$^{32}$P]dCTP was purchased from New England Nuclear. Other organic and inorganic compounds were purchased from Sigma or USBC.

**Growth and storage of bacterial strains.** *S. pneumoniae* strains were grown without aeration in a complex broth medium (CAT) or embedded in agar solidified CAT in Petri plates. 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. This mixture was autoclaved and cooled to 60°C and then supplemented aseptically with 0.5 % (w/v) glucose and 0.016 M $K_2HPO_4$. For solid CAT medium, 1.5 % (w/v) of agar was added prior to autoclaving. Growth of

pneumococcus in broth was monitored by spectrophotometry at 550 nm in 13 mm tubes. Based on calibration of the spectrophotometer used, an $OD_{550nm}$ of 0.2 corresponds to about 2 x $10^8$ colony forming units (CFU) per ml. Pneumococcal strains were routinely grown to a cell density of 2 x $10^8$ CFU/ml for immediate use or for storage in 10 % (v/v) glycerol at -80°C for future use as they tend to autolyse if grown to higher cell densities. All strains of E. coli were routinely grown at 37°C in Luria-Burtani (LB) broth with aeration or on the surface of LB agar in Petri dishes. LB medium contained 10 g casein hydrolysate, 5 g yeast extract, and 10 g NaCl per liter and was adjusted to pH 7.5 with NaOH. For solid medium, 2.0 % (w/v) agar was added prior to autoclaving. Broth cultures of E. coli were grown for a minimum of 16 hours to stationary phase. Sterile glycerol was added to a broth culture to a final concentration of 50 % (v/v) for storage at -20°C.

E. faecalis was grown at 37°C in CAT broth without aeration for a minimum of 16 hours and stored at -80°C with the addition of 10 % (v/v) glycerol. E. faecalis was also grown on the surface of CAT agar in Petri dishes at 37°C. Occasionally, E. faecalis was grown embedded between layers of CAT agar.

All selective antibiotic concentrations as they apply to the different strains used are listed in Table 3.

TABLE 3

SELECTIVE ANTIBIOTIC CONCENTRATIONS

| Genotype or 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 | 20 |
| *str* | streptomycin | 200 | 200 |
| *tet* (Tn*5253*) | tetracycline | 2 | 5 |
| *Enterococcus faecalis* | | | |
| $Cm^r$ | chloramphenicol | 25 | 75 |
| $Fus^r$ | fusidic acid | 25 | 50 |
| $Rif^r$ | rifampicin | 25 | 50 |
| $Str^r$ | streptomycin | 200 | 1000 |
| $Tc^r$ | tetracycline | 4 | 12 |
| *Escherichia coli* | | | |
| $Amp^r$ | ampicillin | 40 | |
| $Cm^r$ | chloramphenicol | 10 | |
| $Em^r$ | erythromycin | 200 | |
| $Tc^r$ | tetracycline | 10 | |

**Preparation of chromosomal DNA.** Pneumococcal cells were grown in broth culture to a density of 2 x $10^8$ CFU per ml. Prior to harvesting the cells, EDTA was added to the cultures to a final concentration of 10 mM and swirled at 0°C for 10 minutes. The cells were harvested by centrifugation in 250 ml polypropylene bottles in a Sorvall GSA rotor at 7,000 RPM at 4°C for 10 minutes. After discarding the supernatant, the pellet was washed by resuspending in SSC (150 mM NaCl, 15mM sodium citrate) and pelleted again as described above. This washed pellet of cells was resuspended in 3 ml lysing solution of 30 mM EDTA, 0.1 % (w/v) sodium dodecyl sulfate (SDS), 0.1 % (w/v) sodium deoxycholate (DOC) and 0.1 % (v/v) diethyl pyrocarbonate. The suspension was incubated at 37°C for 15 minutes or until visible lysis occurred at which time it was held at 65°C for another 15 minutes. This crude lysate appeared almost colorless and very viscous. The lysate was used to prepare purified chromosomal DNA by the method of Marmur (27) and stored in TE buffer (10 mM Tris-HCL, 1 mM EDTA, pH 8.0) at 4°C or -20°C. For some purposes, a crude lysate was prepared with 0.01 % SDS and 0.01 % DOC, diluted 10-fold with SSC and used as donor DNA in transformation.

Lysates of E. faecalis were prepared in the following manner. Cells from a 10 ml culture were harvested by centrifugation at 8,000 RPM at 4°C for 10 minutes in 15 ml Corex tubes in a Sorvall SA-600 rotor.

The pellet was washed in SSC once, resuspended in 1 ml of SSC and transferred to an Eppendorf tube. The cells were pelleted again at 10,000 RPM for 4 minutes at room temperature in a microfuge. After removing the supernatant, the pellet was resuspended in 100 $\mu$l of a solution containing 25 % (w/v) sucrose, 50 mM Tris-HCl, pH 8.0. To this, 20 $\mu$l of a lysozyme solution was added. The lysozyme solution contained 5 mg/ml lysozyme, 30 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 50 mM NaCl. This mixture was incubated at room temperature for 15 minutes. Next, 20 $\mu$l of 0.5 M EDTA was added and the tube was held at room temperature for 15 minutes. These protoplasts were lysed with the addition of 180 $\mu$l of sarkosyl solution which contained 2 % (w/v) sarkosyl, 30 mM Tris-HCl (pH 8.0), 5 mM EDTA (pH 8.0), and 50 mM NaCl. After 15 minutes incubation at room temperature, this lysate was used to isolate purified chromosomal DNA.

Chromosomal DNA from lysates was purified by the method of Marmur (27) and stored in TE buffer at 4°C or -20°C. DNA prepared in this manner was suitable for transformation, restriction endonuclease digestion, and blot hybridization.

**Preparation of plasmid DNA.** E. coli strains were grown to stationary phase under appropriate selection without amplification. Plasmid DNA was then isolated by rapid alkaline-sodium dodecyl sulfate extraction (2,10). For highly purified plasmid DNA, extraction was followed

by sedimentation on cesium chloride-ethidium bromide density gradients.

**Transformation.** Frozen competent recipient cultures of pneumococcus were prepared by methods described by Guild and Shoemaker (15) and stored in aliquots at -80°C. Competent cells were thawed on ice, mixed with donor DNA and incubated at 37°C for 30 minutes. DNase I was added to a final concentration of 10 μg/ml and the mixture was maintained at 37°C for 5 minutes. The cells were diluted and plated without selection by an agar overlay method. The plates were then overlaid with selective agar after 90 minutes incubation at 37°C for phenotypic expression. Plates were then incubated at 37°C overnight.

Methods described by Hanahan (17) were used to transform E. coli with plasmid DNA. Transformed cells were diluted and plated on selective LB plates. Where appropriate, 5-bromo-4-chloroindolyl-ß-D-galactopyranoside (X-gal) was used for phenotypic differentiation of transformants.

**Agarose gel electrophoresis.** DNA samples were separated by electrophoresis at room temperature on horizontal agarose gels using TBE buffer (89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.3). Gels were stained for 10 minutes at room temperature in deionized water containing 1 μg/ml ethidium bromide and destained in deionized water for 10 minutes. Fragment sizes were estimated by comparison to molecular weight standards:

*Hin*dIII fragments of lambda phage and *Hae*III fragments of $\phi$X174 phage. Agarose gels of 0.8 % (w/v) were used for most purposes. Occasionally, 0.6 % gels were used to resolve large fragments and smaller fragments (less than 2.0 kb) were separated using 1.2 % gels.

**DNA-DNA hybridization.** DNA samples for blot hybridizations were transferred from agarose gels to GeneScreen Plus (New England Nuclear) membranes according to manufacturer's recommendations. Recombinant clones were identified by colony hybridization after transfer of colonies to Colony/Plaque Screen (New England Nuclear) nitrocellulose membranes according to the method described by Maniatis, et al. (26). Probes used in hybridizations were radioactively labeled by nick translation with [$\alpha$-$^{32}$P]dCTP according to standard methods (26). Salmon sperm DNA (Sigma) was dissolved in TE buffer, mechanically sheared using a French press, extracted with phenol:chloroform:isoamyl alcohol (25:24:1) then filter sterilized prior to use. Not more than 5 x $10^6$ CPM of labeled probe DNA was used for each hybridization to obtain significant signal over noise. After hybridization at 65°C for 18 hours, the filters were washed, dried and exposed to Kodak X-OMAT AR film at -80°C.

**Conjugation.** Conjugation and scoring for transconjugants between pneumococcal donors and recipients was performed as described (37). The donor

and recipient cells were mixed at a ratio of 1:10 (donors:recipients) in the presence of DNase I (100 $\mu$g/ml). The mixture was filtered onto sterile 13-mm nitrocellulose filters. The filters were then placed cell-side-down onto CAT agar containing 10 mM $MgSO_4$, BSA (2 mg/ml), and DNase I (100 $\mu$g/ml), overlaid with the same agar as the base layer, and incubated at 37°C for 4 hours. After incubation, the filter and surrounding agar were removed and washed in CAT broth containing 10 mM $MgSO_4$, BSA (2 mg/ml) and DNase I (10 $\mu$g/ml). The wash was decanted, diluted and plated to score for donors, recipients and transconjugants by agar overlay plating.

This same procedure was used in conjugation between pneumococcal donors and _E. faecalis_ recipients except that the conjugation was allowed to continue for 18 hours. _E. faecalis_ transconjugants were scored by direct plating on the surface of selective media rather than by the agar overlay method used in selection of pneumococcal transconjugants. It was observed that the use of Difco Bacto-casitone rather than USBC brand casein hydrolysate greatly enhanced the frequency of conjugation between pneumococcal donors and _E. faecalis_ recipients. Unselected markers among transconjugants were scored for by replica plating with sterile toothpicks onto selective media containing 2 % bovine whole blood.

# CHAPTER III

## RESULTS

The recent observation that Tn5253 contained a smaller, independent conjugative transposon, Tn5251, demanded the reevaluation of the hypothesis that a Tn916-like element was the progenitor of all streptococcal conjugative transposons. The observed structural and functional features of Tn5251 were similar to those of Tn916 and Tn1545 and were distinctly different from those of the parental element, Tn5253. Although Tn5251 is homologous to Tn916, the terminal sequences of Tn5253 contain no detectable homology to Tn916. If conjugative properties of Tn5253 were due to the presence of Tn5251, then Tn5251, by some unknown mechanism, must have attained the ability to recognize the terminal ends of Tn5253 to initiate transfer and lost the ability to recognize its own terminal sequences. However, this seemed unlikely. Together, these data have raised questions regarding the functional role, if any, of Tn5251 in the transposition of Tn5253. To clarify these seemingly paradoxical observations, one must first remove the Tn5251 sequences from within Tn5253 and study the properties of the remaining sequences.

One of the ways this could be accomplished is

through restriction endonuclease digestion of a recombinant plasmid derived from Tn5253 which also contains the Tn5251 segment. After digestion, those fragments containing the Tn5251 sequences could be removed and the remaining sequences, now devoid of the Tn5251 segment, could be ligated together. However, this is dependent on the availability of restriction sites close to the termini of Tn5251 and could result in the simultaneous removal of some flanking sequences outside of Tn5251. An alternate method which allowed the retention of all of the sequences outside of Tn5251 was chosen in this study.

To accomplish this goal, we exploited the observation that Tn5251 was unstable when present on the E. coli plasmid, pVJ403, and excised in the absence of selective pressure. The deletion event resulted in the fusion of the sequences which previously flanked the transposon. This fusion was expected to almost restore the target site which was present before the insertion of Tn5251. This assumption that the sequence of this site before the insertion and after the excision of Tn5251 was preserved relied primarily on the observed behavior of a related element, Tn916. Previous studies of Tn916 have shown that insertion and excision of this transposon did not result either in any increase or decrease of the bases at the target site. The only change that occurred was the replacement of five bases without altering the

reading frame. Based on the structural similarities, we assumed that Tn916 and Tn5251 were also mechanistically similar.

## In vivo deletion of Tn5251 from Tn5253

**Generation of pVJ403Δtet.** A 23 kb XbaI fragment from the central region of Tn5253 carrying the Tn5251 sequences has been cloned into the XbaI site of the plasmid vector, pVA891, to generate the recombinant plasmid, pVJ403 (Figure 2) (1). The tet determinant on the passenger DNA of pVJ403 confers tetracycline resistance (4 μg/ ml) to E. coli cells harboring this plasmid. In E. coli DH5α, pVJ403 is stable if maintained under tetracycline selection. However, in the absence of selective pressure, tetracycline sensitive cells arose at a detectable frequency after overnight growth. Of the two hundred transformants screened by replica-plating, twenty-three colonies had lost resistance to tetracycline. Analysis by agarose gel electrophoresis of the plasmid DNA from six of these $Tc^s$ isolates revealed the loss of an 18 kb segment of DNA in each case. In each case, restriction mapping of the $Tc^s$ derivatives of pVJ403 localized the deletion of an 18 kb segment between the HindIII site at coordinate 3.0 and the BamHI site at coordinate 21.8 in the passenger DNA producing a 0.8 kb fusion fragment flanked by sites for HindIII and BamHI (Figure 2). This deletion represented the loss of Tn5251

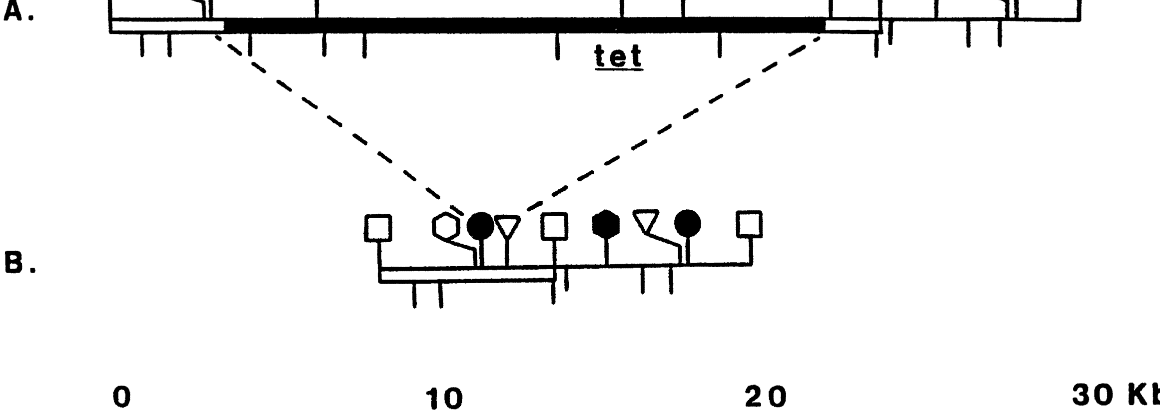


Figure 2. Restriction endonuclease map of pVJ403 and the deletion derivative, pVJ403Δtet. A, pVJ403; B, pVJ403Δtet; , XbaI; , KpnI; , BamHI; , HindIII; , EcoRI; , HincII. The shaded region indicates the location of Tn5251 which spontaneously excises in E. coli in the absence of tetracycline selection. The tet determinant located within Tn5251 is also indicated.

from pVJ403 and splicing of the flanking sequences to create a 5.2 kb fusion fragment of passenger DNA and tetracycline sensitivity. This new plasmid, termed pVJ403Δtet, served as a naturally created tool to introduce the deletion of Tn5251 in cells carrying the intact Tn5253.

**In vivo deletion of Tn5251 from Tn5253.** In pneumococcal transformation, double stranded donor DNA is rendered single stranded upon entry (22). If the donor DNA is homologous to the chromosomal DNA of the recipient, the donor molecule pairs with the recipient chromosome at regions of homology leading to genetic recombination. Insertions or deletions carried by the donor DNA could be introduced into the recipient genome provided there exists sufficient homology flanking the insertion or deletion site to allow efficient pairing between the donor and recipient DNA. The sequences flanking the insertion or deletion in the donor DNA will base pair with homologous sequences in the recipient genome displacing the complementary strand in the recipient cell. The unpaired intervening sequences either in the donor or recipient would loop out and after one round of replication, the heteroduplex would be resolved. As a result, one of the two daughter cells would remain unchanged while the other would be expected to contain the donor DNA carrying the desired change. pVJ403Δtet contained the desired deletion of Tn5251 and

thus was used to induce the in vivo deletion of Tn5251 from Tn5253. Figure 3 diagramatically illustrates this strategy employed to induce the deletion of the Tn5251 sequences from Tn5253.

The plasmid pVJ403Δtet was digested with XbaI. The resulting fragments were separated by agarose gel electrophoresis and the 5.2 kb fusion fragment of passenger DNA was isolated by electroelution. The purified fragment was introduced into competent DP1324 cells, carrying the entire Tn5253 element and a chromosomal point mutation conferring resistance to streptomycin ($Str^r$), via transformation. Restriction mapping had shown that the 5.2 kb fusion fragment contained at least 3.0 kb of DNA to the left and 1.4 kb of DNA to the right of the Tn5251 element, thus carrying sufficient homology to allow efficient genetic pairing between the regions flanking the tet segment and to introduce the expected deletion. Transformants carrying the deletion were expected to be tetracycline sensitive. After transformation, the cells were incubated for two hours without selection to permit phenotypic expression. This period of time was expected to allow resolution of the heteroduplex and segregation of the $Tc^r$ and $Tc^s$ diplococcal daughter cells. The mixture of cells was diluted and scored on nonselective plates. The following day, four thousand colonies were screened for str, Tc and Cm resistance by replica-plating on selective blood agar

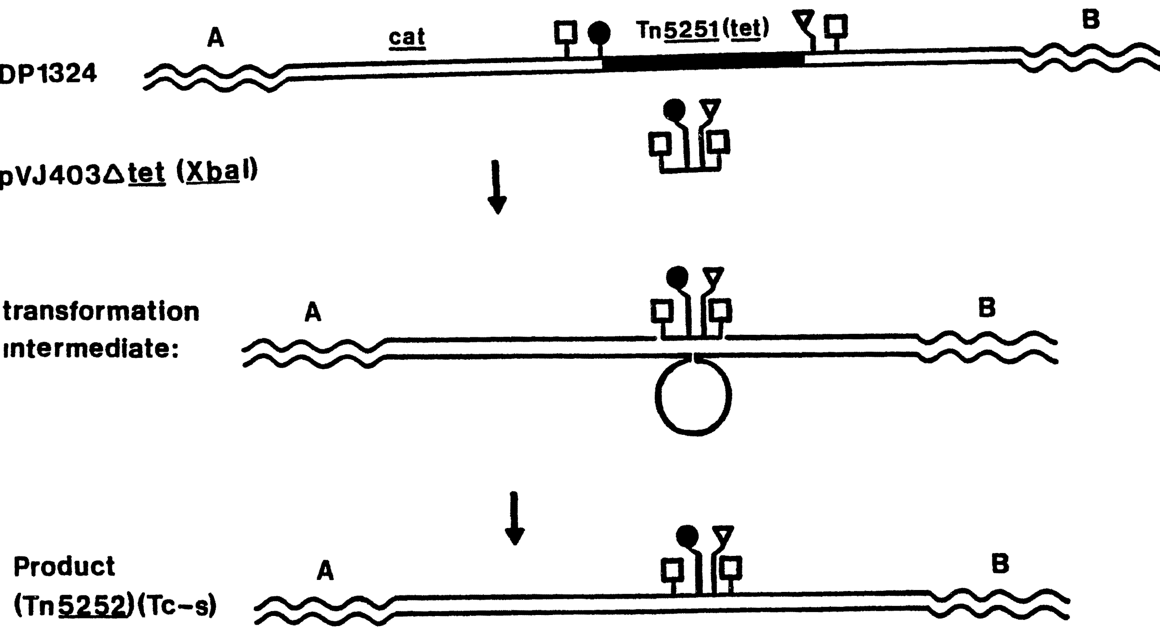


Figure 3. Strategy for the in vivo deletion of Tn5251 from Tn5253. ═══ , Tn5253 DNA; ≈≈ , pneumococcal chromosomal DNA; A and B refer to sequences flanking the left and right termini of Tn5253 respectively; ▬ , Tn5251 DNA; ⍞ , XbaI; ⚲ , HindIII; ∇ , BamHI. The location of the cat and tet determinants are also indicated. The 5.0 kb fusion fragment from pVJ403Δtet carrying the sequences flanking Tn5251 in Tn5253 was used as donor DNA to transform DP1324. The donor DNA was taken into the recipient cell as a single stranded molecule. As shown in the transformation intermediate, the donor DNA was expected to base pair with the homologous sequences, displacing one of the existing strands, and inducing the Tn5251 sequences to loop out as indicated. After replication and segregation, transformants were identified as $Tc^S$ isolates.

plates. Thirty str$^r$, Cm$^r$, Tc$^s$ colonies were isolated and designated TA1 through TA30.

**Genetic analysis of Tc$^s$ isolates by transformation.** The chloramphenicol resistance determinant in Tn5253 (cat) is flanked by direct repeats of 3 kb. Genetic recombination events occurring between these direct repeats can result in the spontaneous loss of approximately 8 kb of DNA spanning the cat region resulting in sensitivity to chloramphenicol (38). DP1333, carrying Tn5253, has lost the cat determinant in this manner and, as a result, is sensitive to chloramphenicol. In addition, DP1333 has acquired a tet-3 point mutation within the tet determinant conferring sensitivity to tetracycline (38). Donor DNA derived from the tet region of Tn5253 is able to transform DP1333 cells giving rise to Tc$^r$ transformants. Similarly, if the tet determinant in Tn5253 contains a point mutation other than the tet-3 point mutation in DP1333, this DNA is also able to transform DP1333 giving rise to Tc$^r$ transformants. However, if the tet determinant in Tn5253 is not present and this DNA is used to transform DP1333, Tc$^r$ transformants could not be recovered due to the lack of the tet sequences.

Crude lysates of ten Tc$^s$ Cm$^r$ isolates (TA1 through TA10) were prepared and used as donor DNA in transformation of DP1333 to determine if the observed sensitivity was associated with a point mutation within

the tet determinant or a deletion of the tet determinant. As an internal control, each sample was also scored for $Cm^r$ transformants. As shown in Table 4, $Cm^r$ transformants were identified while $Tc^r$ transformants were not observed. This provided a strong indication that the tet determinant within Tn5253 was absent from these isolates while the cat determinant remained.

**Physical analysis of $Tc^s$ isolates by Southern hybridization.** Four representative isolates (TA1, TA2, TA3, and TA4) were selected for further analysis to confirm the deletion of Tn5251 from within Tn5253. Chromosomal DNAs from each of the four isolates and the controls, Rx1 and DP1322, were digested with EcoRI or HindIII. The restriction fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose membrane and probed with $^{32}P$-labeled pVJ403. As expected, wild type Rx1 DNA, which does not contain Tn5253, did not react with the probe (Figure 4, lane a). Due to the presence of a single site, EcoRI cleaves the Tn5251 segment within Tn5253 into two fragments of 23 kb and 12.2 kb. Further, three HindIII fragments of 12.6 kb, 8.5 kb, and 3.6 kb from Tn5253 were expected to react with the probe. As expected, the probe reacted with two EcoRI fragments of 23 kb and 12.2 kb and three HindIII fragments of 12.6 kb, 8.5 kb, and 3.1 kb of DP1322 DNA, carrying the entire Tn5253 element (Figure 4, lane b). However, in all four $Tc^s$ isolates tested, the

TABLE 4

TRANSFORMATION OF DP1333 WITH CRUDE LYSATES OF TEN $TC^S$ ISOLATES

| Donor DNA | Transformants/ml | |
|---|---|---|
| | $Cm^r$ | $Tc^r$ [c] |
| TA1 | $8.0 \times 10^4$ | $< 2 \times 10^3$ |
| TA2 | $1.1 \times 10^5$ | $< 2 \times 10^3$ |
| TA3 | $1.3 \times 10^5$ | $< 2 \times 10^3$ |
| TA4 | $7.7 \times 10^4$ | $< 2 \times 10^3$ |
| TA5 | $7.3 \times 10^4$ | $7.5 \times 10^3$ |
| TA6[a] | $7.4 \times 10^4$ | $7.0 \times 10^3$ |
| TA7 | $3.5 \times 10^4$ | $4.0 \times 10^3$ |
| TA8 | $1.3 \times 10^5$ | $7.5 \times 10^3$ |
| TA9 | $6.0 \times 10^4$ | $5.5 \times 10^3$ |
| TA10 | $5.9 \times 10^4$ | $4.5 \times 10^3$ |
| DP1322[b] | $1.0 \times 10^5$ | $1.4 \times 10^6$ |

a TA6 lysate gave $1.3 \times 10^6$ $Str^r$ transformants/ml of DP1333.

b DP1322 DNA was used as a positive control at 1 μg/ml of DNA and gave $1.3 \times 10^6$ $Str^r$ transformants/ml of DP1333.

c The frequency of spontaneous reversion of <u>tet</u>-3 to tetracycline resistance is about $10^{-4}$.

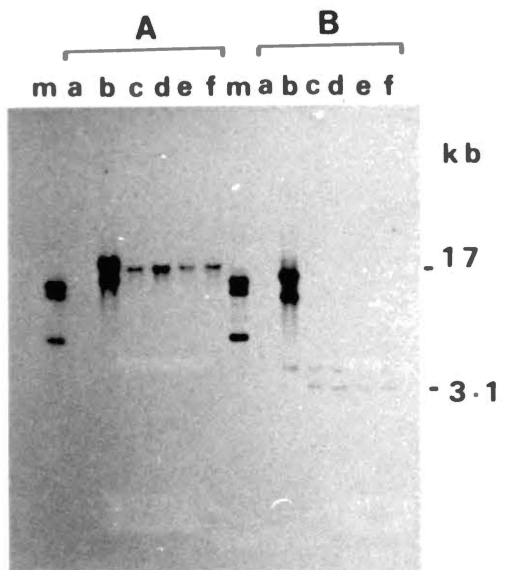


Figure 4. Autoradiogram of Southern hybridization showing the in vivo deletion of Tn5251 from Tn5253. $^{32}$P-labeled pVJ403 was used as a probe in hybridization with (A) EcoRI and (B) HindIII digests of chromosomal DNA from (a) Rx1, (b) DP1322, and (c through f) four Tc$^{s}$ transformants, (m) molecular weight standard.

probe reacted with a single 17 kb EcoRI fragment and with two HindIII fragments of 3.1 kb and 3.6 kb (Figure 4, lanes c through f). The deletion of the 18 kb Tn5251 segment from within Tn5253 would result in the loss of the EcoRI site within Tn5251 and the remaining EcoRI fragment size expected to react with the probe totals 17 kb. In addition, the two HindIII fragments of 12.6 kb and 8.5 kb would be reduced to a single fragment of 3.1 kb while the 3.6 kb fragment would remain unaltered if the 18 kb Tn5251 segment is removed from within Tn5253. These results demonstrate that the 18 kb Tn5251 was deleted in vivo from within Tn5253 in each case. One of these $Tc^{S}$ deletion mutants was selected for further study and designated SP1000.

**Conjugative Transfer Properties of Tn5253ΔTn5251**

**Intraspecific conjugative transfer of Tn5253ΔTn5251.** As described earlier, spontaneous curing of the cat region in Tn5253 could occur at a detectable frequency due to the flanking direct repeats resulting in sensitivity to chloramphenicol. With the deletion of the tet determinant from Tn5253, the only other determinant one can phenotypically score for is cat. To facilitate phenotypic selection of transconjugants in filter mating, a second genetic marker was artificially introduced into Tn5253ΔTn5251. The recombinant plasmid, pVJ91, contains passenger DNA derived from the right end of Tn5253 on the

plasmid vector pVA891. Although this plasmid lacks a pneumococcal replicon, it can express erythromycin (Em) resistance in pneumococcus when inserted into the chromosome. When introduced by transformation into pneumococcal cells containing Tn5253, the passenger DNA in pVJ91 was expected to pair with the region of homology within Tn5253. This pairing was expected to be followed by genetic recombination and insertion of pVA891 into the element conferring resistance to erythromycin. Insertion of pVA891 into this region was known not to affect the transfer of the parental element. Competent SP1000 and DP1324 cells were transformed with pVJ91 donor DNA and scored for $Em^r$ transformants. One $Em^r$ transformant of SP1000 was selected for conjugation studies and designated SP1001. In addition, one $Em^r$ transformant of DP1324 was designated DP1327.

Each of these $Em^r$ strains isolated were used as a donor in conjugation by filter-mating with DP1002 (nov-1) recipients. Initially, difficulties with the isolation of transconjugants were encountered. Transconjugants were undetectable due to a lawn of confluent growth on selective plates. This problem was resolved by plating smaller volumes of the conjugation mixtures to dilute the cells. Usually 100 $\mu$l to 200 $\mu$l of each filter wash was plated to obtain approximately $10^7$ total CFU per plate. In addition, drugs selective for the recipient chromosomal markers were included in all the agar layers

except the base layer. After allowing sufficient incubation period for phenotypic expression, the final drug layer was added which contained antibiotics selective for markers of the conjugative element in addition to the drugs selective for markers of the recipient. This reduced the growth of cells that arose from spontaneous mutation of the donor cells to the recipient phenotype. Combined, these changes reduced the amount of background growth and significantly improved the detection of transconjugants.

As shown in Table 5, Cm and Em transferred from SP1001 to DP1002 at a frequency of $3.0 \times 10^{-7}$ $Cm^r$ $Em^r$ $Nov^r$ transconjugants per donor and from DP1327 to DP1002 at a frequency of $9.0 \times 10^{-7}$ $Cm^r$ $Em^r$ $Nov^r$ transconjugants per donor. The transfer frequency of markers derived from the element was at least one order of magnitude higher than the transfer of the chromosomal markers. This provided a strong indication that the elements from SP1001 or DP1327 mobilized into the recipient cells by a conjugative process and not by transformation. One DP1002 transconjugant harboring Tn<u>5253</u>ΔTn<u>5251</u> was isolated and designated SP1002.

This new isolate, SP1002 (<u>nov</u>-1, Tn<u>5253</u>ΔTn<u>5251</u>, Em), was then used as a donor in conjugation with DP1004 (<u>str</u>-1) recipients. In this mating, Cm and Em transferred to the recipient strain at a frequency of $5.7 \times 10^{-6}$ which was comparable to that of the control

TABLE 5

TRANSFER OF Tn5253ΔTn5251 TO DP1002 BY FILTER-MATING

| Donor | Viable count (per ml) | | $Em^r$ $Cm^r$ $Nov^r$ transconjugants per donor |
|---|---|---|---|
| | Donor | Recipient[a] | |
| SP1001[b] | $1.8 \times 10^8$ | $2.5 \times 10^8$ | $3.0 \times 10^{-7}$ |
| DP1327[c] | $1.5 \times 10^8$ | $4.7 \times 10^8$ | $9.0 \times 10^{-7}$ |

a In each case, the recipient strain was DP1002.

b $5.0 \times 10^{-8}$ $Nov^r$ $Str^r$ transformants per donor were seen.

c $6.7 \times 10^{-8}$ $Nov^r$ $Str^r$ transformants per donor were seen.

mating (Table 6). Again, conjugative transfer of the $Cm^r$ and $Em^r$ markers from the element was indicated based on comparison to transfer of the chromosomal markers which was ten-fold less efficient. Based on these results, we concluded that Tn5253ΔTn5251 was capable of conjugative transfer and this novel element was designated Tn5252.

**Target preference of Tn5252 in pneumococcus.** As previously stated, Tn5253 inserts at a preferred target site in the pneumococcal chromosome following conjugal transfer. To determine whether Tn5252 also carried this property, three transconjugants were selected for further study to examine the target preference of Tn5252 in pneumococcus following transfer by conjugation. Chromosomal DNAs from these three transconjugants, DP1322, and Rx1 were purified, digested with EcoRI, separated by agarose gel electrophoresis and transferred to a nitrocellulose membrane for Southern hybridization. The recombinant plasmid, pVJ183, carrying a 5.4 kb PstI fragment containing the target site for Tn5253 in the Rx1 chromosome, on the plasmid vector, pVA891, was used as a probe. In Rx1, the probe hybridized to two EcoRI fragments of 3.8 kb and 2.1 kb as expected (Figure 5). The 2.1 kb EcoRI fragment contains the target site for Tn5253 in Rx1 which is disrupted upon insertion of the element into the Rx1 chromosome following conjugative transfer. As expected, in DP1322, carrying Tn5253, the 2.1 kb EcoRI fragment disappeared and was replaced with

TABLE 6

CONJUGATION BY FILTER-MATING FROM SP1002 DONORS

| Donor | Viable count (per ml) | | $Em^r$ $Cm^r$ transconjugants per donor |
|---|---|---|---|
| | Donor | Recipient | |
| SP1002[a] | $3.5 \times 10^7$ | $7.3 \times 10^7$ [b] | $5.7 \times 10^{-6}$ [c] |
| DP1327[d] | $3.4 \times 10^7$ | $1.2 \times 10^8$ [e] | $9.9 \times 10^{-6}$ [f] |

a  $2.9 \times 10^{-7}$ $Nov^r$ $Str^r$ transformants per donor were seen.

b  The recipient strain was DP1004 ($Str^r$)

c  Transconjugants were identified as $Em^r$ $Cm^r$ $Str^r$ $Nov^s$.

d  $5.8 \times 10^{-7}$ $Nov^r$ $Str^r$ transformants per donor were seen.

e  The recipient strain was DP1002 ($Nov^r$)

f  Transconjugants were identified as $Em^r$ $Cm^r$ $Nov^r$ $Str^s$.

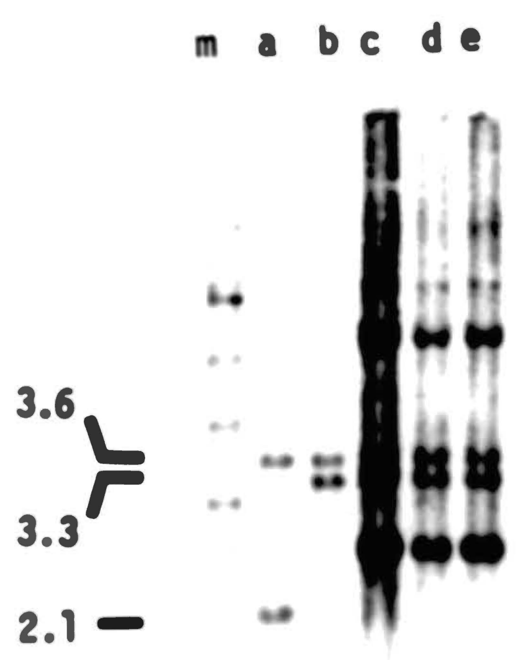


Figure 5. Autoradiogram of Southern hybridization showing target preference of Tn5252 in pneumococcus following conjugative transfer. $^{32}$P-labeled pVJ183 was used as a probe in hybridization with EcoRI digests of chromosomal DNA from (a) Rx1, (b) DP1322, and (c through e) three Rx1 transconjugants carrying Tn5252, (m) molecular weight standard.

two fragments of 3.6 kb and 3.3 kb while the EcoRI fragment 3.8 kb was retained. In the three transconjugants of Tn5252 analyzed, the probe reacted with five EcoRI fragments. Three of these fragments were of the same size as those in DP1322 (3.8 kb, 3.6 kb, and 3.3 kb). The 2.1 kb EcoRI fragment observed in Rx1 was again replaced in the Tn5252 transconjugants with two fragments of 3.6 kb and 3.3 kb indicating that, in the transconjugants analyzed, Tn5252 preferred the same target site in Rx1 as Tn5253. As previously mentioned, pVA891 was inserted into the right end of Tn5252 to create SP1001. This region within Tn5252 containing pVA891 was expected to react with the vector portion of the probe producing two additional EcoRI fragments of 6.9 kb and 2.9 kb in the Tn5252 transconjugants. As expected, the probe reacted with two additional EcoRI fragments of this size in the Tn5252 transconjugants (Figure 5, lanes c through e).

**Interspecific conjugative transfer of Tn5252.** To determine whether Tn5252 was capable of interspecific conjugal transfer, SP1000 was used as a donor in filter-mating with E. faecalis UV202 recipients. E. faecalis is heterologous to pneumococcus and, in contrast to pneumococcus, is incapable of natural transformation; therefore, mobilization of Tn5252 into E. faecalis was expected to show the capacity for interspecific transfer and also rule out the possibility that the transfer was

due to transformation. In addition, the recipient strain *E. faecalis* UV202 is *rec* deficient. Thus, mobilization of Tn*5252* or Tn*5253* into this recipient strain was expected to demonstrate their independence from host recombination functions in conjugal transfer. *E. faecalis* UV202 also contains two chromosomal point mutations conferring resistance to rifampicin ($Rif^r$) and fusidic acid ($Fus^r$).

Several problems were encountered in the selection of transconjugants during the initial conjugation experiments. These difficulties were resolved when small volumes of the filter wash were plated directly on the surface of selected media to obtain approximately $10^8$ total CFU per plate. This modification enhanced selection of *E. faecalis* transconjugants as these conditions were unfavorable for the growth of the anaerobic, pneumococcal donors. Filter-mating between DP1324, carrying the entire Tn*5253* element, and UV202 was also performed to provide a control. $Cm^r$ transferred from SP1000 to UV202 at a frequency of $1.0 \times 10^{-6}$ $Cm^r$ transconjugants per donor. In the control mating, Tn*5253* transferred from DP1324 to UV202 at a frequency of $1.2 \times 10^{-5}$ $Cm^r$ transconjugants per donor (Table 7). $Str^r$ $Rif^r$ transformants were not observed with any of the mating pairs indicating that the markers mobilized into the UV202 recipient cells by conjugation and not by transformation. Eight UV202 transconjugants were

TABLE 7

TRANSFER OF Tn5252 TO E. FEACALIS UV202
BY FILTER-MATING

| Donor | Viable count (per ml) | | $Cm^r$ $Rif^r$ $Fus^r$ transconjugants per donor |
|---|---|---|---|
| | Donor | Recipient[a] | |
| SP1000 | $1.0 \times 10^7$ | $1.4 \times 10^9$ | $1.0 \times 10^{-6}$ |
| DP1324 | $1.7 \times 10^6$ | $5.0 \times 10^8$ | $1.2 \times 10^{-5}$ |

a In each case, the recipient strain was UV202.

selected for further studies (Fx21-1 and Fx21-2 where isolated from the control mating with DP1324 donors while Fx21-19, Fx21-20, Fx21-21, Fx27-7, Fx27-8, and Fx27-9 where isolated from matings between SP1000 donors and UV202 recipients).

**Genetic analysis of the UV202 transconjugants by transformation.** Preliminary analysis of the UV202 transconjugants involved transformation studies similar to those employed in the analysis of the potential Tn5253 Tn5252 isolates. The 3 kb direct repeats flanking the cat determinant in Tn5253 and Tn5252 share homology with regions in the Rx1 chromosome (30). Due to this shared homology, donor DNA derived from Tn5253 or Tn5252 can transform DP1333 or the wild type Rx1 giving rise to chloramphenicol resistant transformants. However, donor DNA derived from Tn5253 or Tn5252 contains more extensive homology to DP1333 than to Rx1; therefore, the frequency of $Cm^r$ transformation of DP1333 is an order of magnitude greater than $Cm^r$ transformation of Rx1. Accordingly, chromosomal DNAs from the eight transconjugants and UV202 were isolated and used as donor DNA in transformation with Rx1 or DP1333 recipient cells. Rx1 did not transform for $Rif^r$ using donor DNA from the transconjugants confirming that the strains isolated were *E. faecalis* and not pneumococcus (Table 8). DNA from Fx21-1, Fx21-2 and DP1324 were able to transform DP1333 to $Tc^r$ while DNA isolated from the recipient *E. faecalis*

TABLE 8

ANALYSIS OF THE UV202 TRANSCONJUGANTS BY TRANSFORMATION

| Donor DNA | Rx1 transformants/ml | | DP1333 transformants/ml |
|---|---|---|---|
| | $Rif^r$ | $Cm^r$ | $Cm^r$ |
| Fx21-1[a] | 200 | $1.9 \times 10^4$ | $3.0 \times 10^5$ |
| Fx21-2[b] | 500 | $2.5 \times 10^4$ | $3.5 \times 10^5$ |
| Fx21-19 | 300 | $9.5 \times 10^4$ | $1.1 \times 10^5$ |
| Fx21-20 | 200 | $4.1 \times 10^3$ | $1.1 \times 10^5$ |
| Fx21-21 | 200 | $3.5 \times 10^3$ | $9.5 \times 10^4$ |
| Fx27-7 | 200 | $4.7 \times 10^3$ | $8.7 \times 10^4$ |
| Fx27-8 | 300 | $3.9 \times 10^3$ | $1.0 \times 10^5$ |
| Fx27-9 | 500 | $2.0 \times 10^4$ | $3.4 \times 10^5$ |
| UV202[c] | 300 | < 100 | < 100 |
| DP1324[d] | n.t. | $2.2 \times 10^4$ | $2.0 \times 10^5$ |

a With DNA from Fx21-1, transformation of Rx1 to $Tc^r$ was not detected while DP1333 was transformed for $Tc^r$ yielding $1.4 \times 10^6$ $Tc^r$ transformants per ml.

b Using DNA from Fx21-2, transformation of Rx1 to $Tc^r$ was not detected while DP1333 was transformed for $Tc^r$ yielding $1.4 \times 10^6$ $Tc^r$ transformants per ml.

c Neither Rx1 nor DP1333 were transformed for $Tc^r$ using DNA isolated from UV202.

d As a control, 1 $\mu$g/ml of DNA from DP1324 was used in transformation of Rx1 and DP1333. $Tc^r$ transformants of DP1333 arose ($1.4 \times 10^6$ $Tc^r$ transformants per ml) while transformation for $Tc^r$ in Rx1 was not detected.

UV202 could not indicating that the tet determinant was present in the former transconjugants. In addition, DNA from all the transconjugants and DP1324 transformed DP1333 to $Cm^r$ at an efficiency approximately ten-fold greater than transformation of Rx1 to $Cm^r$ under similar conditions. Further, UV202 donor DNA did not transform DP1333 or Rx1 for $Cm^r$. These results suggest that Tn5253 was present in Fx21-1 and Fx21-2 and that Tn5252 was present in Fx21-19, Fx21-20, Fx21-21, Fx27-7, Fx27-8, and Fx27-9.

**Physical analysis of the UV202 transconjugants by Southern hybridization.** To test whether the integrity of Tn5252 was maintained following conjugal transfer to *E. faecalis* and to determine its target preference in this host, the chromosomal DNAs from these strains were isolated, digested with restriction endonucleases and used in various Southern hybridization experiments.

To determine whether the target site for Tn5253 in Rx1 is homologous to sequences within other streptococcal species, the chromosomal DNAs from several different strains were probed with pVJ187. The plasmid, pVJ187, carries a 2.1 kb *Eco*RI fragment containing the target site for Tn5253 in Rx1 on the plasmid vector pACYC184. *Eco*RI digests of chromosomal DNAs from Rx1, DP1322, *E. faecalis* JH2-2, *E. faecalis* UV202, *S. agalactiae* ATCC 12386, *S. pyogenes* ATCC 21547, and the UV202 transconjugants were probed with $^{32}$P-labeled pVJ187. As

expected, a 2.1 kb EcoRI fragment from Rx1 reacted with the probe (Figure 6, lane a). Two EcoRI fragments of 3.3 kb and 3.6 kb, representing the right and left junctions of Tn5253 in Rx1, respectively, reacted with this probe in DP1322 (Figure 6, lane c), which carries Tn5253. E. faecalis JH2-2, E. faecalis UV202, and S. agalactiae ATCC 12386 did not carry any detectable homology to the target site for Tn5253 in Rx1 (Figure 6, lanes d through f, respectively) as there was no detectable hybridization. S. pyogenes ATCC 21547 showed very weak homology to the target probe (Figure 6, lane g) revealing an EcoRI fragment of approximately 9.5 kb. Two EcoRI fragments in the Tn5253 transconjugants (8.2 kb and 6.5 kb) and one EcoRI fragment in the Tn5252 transconjugants (8.5 kb in Fx21-19 and 2.4 kb in Fx21-20, Fx21-21, Fx27-7, and Fx27-8) reacted with pVJ187. This homology was very weak when compared to that of Rx1 or DP1322 and required a seven-fold increase in exposure time to detect the reacting bands. This suggested that a small portion of donor target DNA (possibly less than 100 bases) may have been transferred into the recipient.

To confirm that all DNA within Tn5252 had been transferred to E. faecalis UV202 recipients, three different probes were used in separate hybridization experiments with DNA derived from the Tn5252 transconjugants. The following probes were used: (1) pVJ403Δ tet, the deletion derivative of pVJ403, (2)

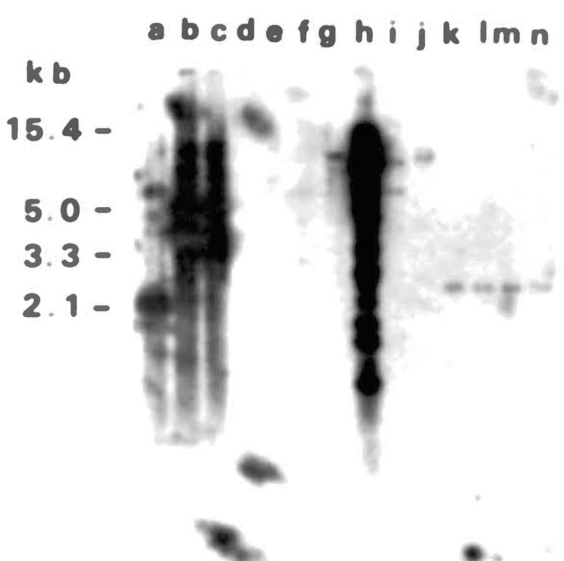


Figure 6. Hybridization between the Tn5253 target in Rx1 and the chromosomal DNA of various streptococcal species. $^{32}$P-labeled pVJ187 was used as a probe in Southern hybridization with EcoRI digests of chromosomal DNA from (a) Rx1, (b) Rx1 (digested with PstI), (c) DP1322, (d) E. faecalis JH2-2, (e) E. faecalis UV202, (f) S. agalactiae ATCC 12386, (g) S. pyogenes ATCC 21547, (h) molecular weight standard, (i) UV202 Tn5253 transconjugant, and (j through n) UV202 Tn5252 transconjugants.

pVJ407, carrying a 2.7 kb KpnI fragment containing the right junction region of Tn5253 in DP1322, in the plasmid vector, pUC19 and (3) a purified 3.6 kb EcoRI fragment containing the left junction region of Tn5253 in DP1322, isolated from the recombinant plasmid, pDP63.

To confirm the presence of DNA internal to the element in the transconjugants, pVJ403Δ tet was used as a probe in hybridization with EcoRI digests of DNA from the transconjugants. As expected, Rx1, which does not contain Tn5253, did not react with the probe (Figure 7, lane a). Two EcoRI fragments of 22.9 kb and 12.2 kb reacted with DNA from the Tn5253 transconjugants and DP1322 indicating that these isolates contained DNA from within Tn5253. The probe reacted with a 17 kb EcoRI fragment in the Tn5252 transconjugants demonstrating the presence of DNA from within Tn5252 in these isolates. Interestingly, the recipient strain, UV202, as well as the UV202 transconjugants consistently showed two EcoRI fragments of 4.9 kb and 4.3 kb reacting with the probe after increased exposure time. In a control experiment, when the vector plasmid, pVA891, was used as a probe, it reacted with two EcoRI fragments of 4.9 kb and 4.3 kb in UV202 and the UV202 transconjugants (data not shown). These observations suggested the presence of homology between the UV202 chromosomal DNA and the vector portion of the probe (that is, pVA891). The erm determinant in pVA891 was derived from a streptococcal source and this

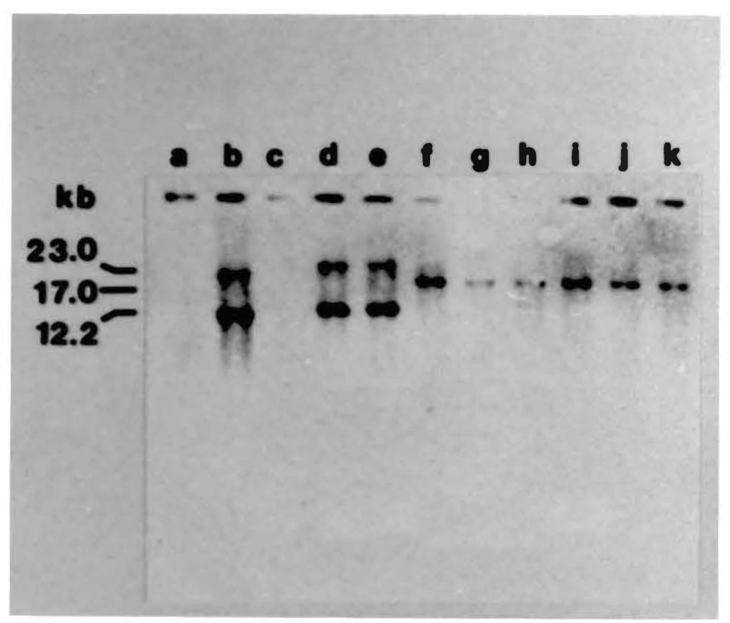


Figure 7. Analysis of the central region of Tn5252 and Tn5253 in E. faecalis UV202 transconjugants. $^{32}$P-labeled pVJ403Δ tet was used as a probe in Southern hybridization with EcoRI digests of chromosomal DNA from (a) Rx1, (b) DP1322, (c) UV202, (d and e) UV202 containing Tn5253 and (f through k) UV202 containing Tn5252.

may explain the homology shared between pVA891 and the E. faecalis UV202 chromosome. To avoid further interference of this type, plasmid probes used in subsequent hybridizations were selected which contained vectors other than pVA891; or, the passenger DNA was purified from the vector portion of the plasmid prior to hybridization.

To examine the right terminus of Tn5253 or Tn5252 in the UV202 transconjugants, $^{32}$P-labeled pVJ407, containing a 2.7 kb KpnI fragment from the right junction of Tn5253 in Rx1, in the plasmid vector pUC19, was used as a probe with EcoRI digests of chromosomal DNA from the UV202 transconjugants. As expected, this probe reacted with two EcoRI fragments in Rx1 of 3.8 kb and 2.1 kb. This 2.1 kb EcoRI fragment in Rx1 contains the target site for Tn5253. Upon insertion of Tn5253 into the Rx1 chromosome, this 2.1 kb EcoRI fragment is disrupted creating two fragments of 3.3 kb and 3.6 kb, representing the left and right junction regions of Tn5253, respectively. This right junction probe was expected to react with a 3.3 kb EcoRI fragment in DP1322, which carries Tn5253. In addition, the 3.8 kb EcoRI fragment reacting in Rx1 with this probe, was expected to remain unchanged in DP1322. As expected, two EcoRI fragments of 3.3 kb and 3.8 kb in DP1322 reacted with this probe. As the UV202 chromosome is heterologous to Rx1, the only fragment expected to react with this probe in the UV202

transconjugants would be a fragment containing the right terminus of Tn5253 or Tn5252. Although the recipient strain, UV202, did not react with this probe (Figure 8), the probe did react with two EcoRI fragments (6.0 kb and 3.8 kb) in the Tn5253 transconjugants. This suggested that two copies of Tn5253 may exist in these transconjugants. A single EcoRI fragment in the Tn5252 transconjugants reacted with the probe indicating that a single copy of Tn5252 may exist within these transconjugants. These EcoRI fragment sizes were of 3.1 kb in Fx21-19 (Figure 8, lane e) and greater that 9 kb in Fx21-20, Fx21-21, Fx27-7, Fx27-8, and Fx27-9 (Figure 8, lanes f through j, respectively).

Chromosomal DNAs from the transconjugants, UV202, Rx1, and DP1322 were then digested with BglII and probed with a purified 3.6 kb EcoRI fragment containing the left junction region of Tn5253 in Rx1 which had been isolated from pDP63. As expected, this probe reacted with two BglII fragments in Rx1 of 4.0 kb and 1.8 kb and three BglII fragments in DP1322 of 4.2 kb, 3.6 kb and 0.45 kb. No detectable homology to UV202 DNA was observed. In contrast, the probe reacted with two BglII fragments of greater than 10 kb and 7.5 kb in chromosomal DNA from the Tn5253 transconjugants and one BglII fragment of greater than 10 kb in chromosomal DNA from the Tn5252 transconjugants (data not shown).

In five of the six Tn5252 transconjugants, each

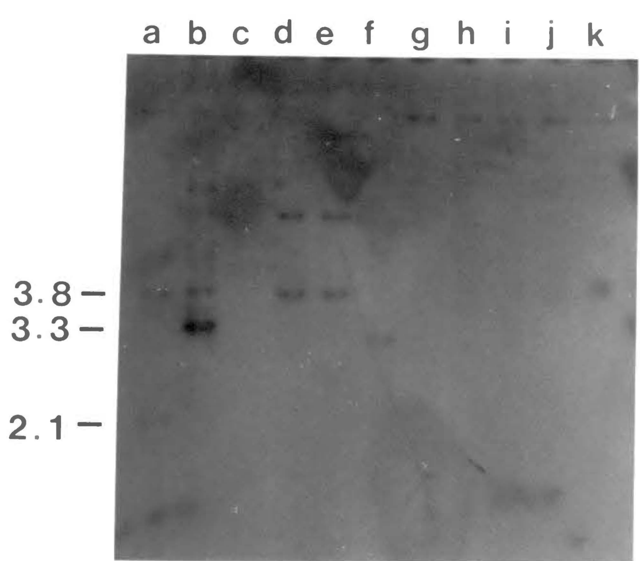


Figure 8. Analysis of the right junction region of *E. faecalis* UV202 transconjugants. $^{32}$P-labeled pVJ407 was used as a probe in Southern hybridization with *Eco*RI digests of chromosomal DNA from (a) Rx1, (b) DP1322, (c) *E. faecalis* UV202 (d and e) *E. faecalis* UV202 Tn*5253* transconjugants, and (f through k) *E. faecalis* UV202 Tn*5252* transconjugants.

junction probe hybridized to fragments of the same size. In contrast, one Tn5252 transconjugant (Fx21-19) seemed to have junction fragments of a different size, suggesting insertion of Tn5252 at a different site in the UV202 chromosome. Interestingly, each probe, from the right or the left junction, reacted with two restriction fragments in the Tn5253 transconjugants. This indicated that two copies of Tn5253 may exist in these transconjugants. These two copies may exist in the chromosome at separate sites or as tandem repeats; or, one copy may exist within the chromosome while the second copy exists as an independent circular molecule. To clarify this, the two transconjugants, Fx21-1 and Fx21-19, harboring Tn5253 and Tn5252 respectively, were more extensively studied by blot hybridization using probes carrying the junction regions. The chromosomal DNAs from DP1322, Fx21-1, and Fx21-19 were digested with BglII, EcoRI, HindIII, KpnI, or PstI and each were probed with DNA from the left or the right junction region of Tn5253 as in DP1322.

pVJ407 was used again as a probe to examine the right junction region of Tn5252 and Tn5253 in the UV202 transconjugants. Chromosomal DNAs from DP1322, Fx21-1, and Fx21-19 were digested with BglII, EcoRI, HindIII, KpnI, or PstI, separated by agarose gel electrophoresis, transferred to a nitrocellulose membrane and probed with $^{32}P$-labeled pVJ407. In each digest, pVJ407 consistently

reacted with two restriction fragments in the Tn5253 transconjugant (Fx21-1) while one restriction fragment was detected in the Tn5252 transconjugant (Fx21-19) (Figure 9). Again, this indicated that two copies of Tn5253 were present in the UV202 Tn5253 transconjugant, while the Tn5252 transconjugant contained only one copy of Tn5252. A complete summary of these results is listed in Table 9.

For a more thorough analysis of the left junction region of Tn5253 and Tn5252 in the UV202 transconjugants, pVJ414, which contains a 3.6 kb EcoRI fragment from the left junction of Tn5253 in DP1322 on the plasmid vector pBluescript SK+, was used as a probe for the left junction region. The passenger DNA in pVJ414 is the same 3.6 kb EcoRI left junction fragment which was purified from pDP63 and used as a probe in the previous hybridization of the left junction region. Using pVJ414 as a probe, two restriction fragments in the Tn5253 transconjugant (Fx21-1) and one fragment in the Tn5252 transconjugant (Fx21-19) were detected in each of the five different restriction digests (Figure 10). A complete summary of these results is listed in Table 10.

Both junction probes consistently hybridized to two restriction fragments in the Tn5253 transconjugant. This confirmed earlier speculations that two copies of Tn5253 exist in the UV202 transconjugant, Fx21-1. Comparison of the restriction fragment sizes reacting with the right

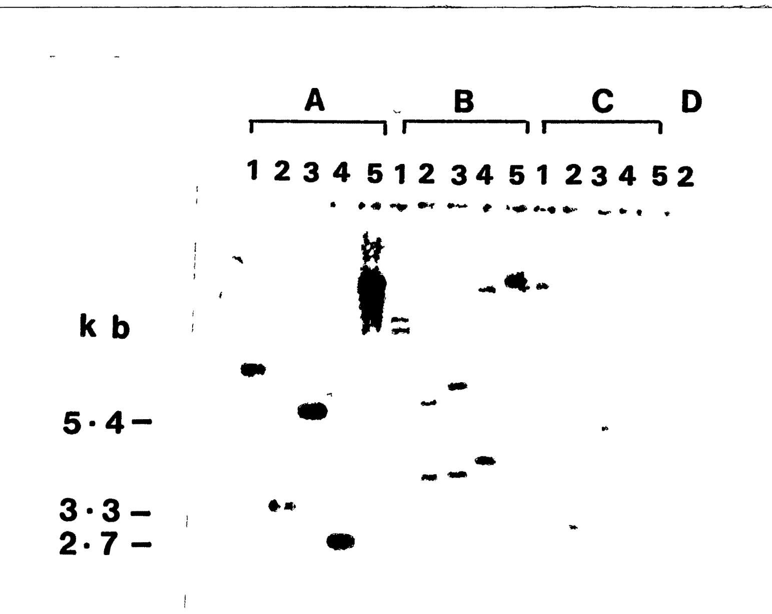


Figure 9. Analysis of the right junction region of two UV202 transconjugants. $^{32}P$-labeled pVJ407 was used as a probe in hybridization with chromosomal DNA from (A) DP1322, (B) Fx21-1, a UV202 transconjugant containing Tn5253, (C) Fx21-19, a UV202 transconjugant containing Tn5252, and (D) *E. faecalis* UV202 digested with (1) *Bgl*II, (2) *Eco*RI, (3) *Hin*dIII, (4) *Kpn*I, and (5) *Pst*I.

TABLE 9

SUMMARY OF RESULTS OF HYBRIDIZATION BETWEEN pVJ407 AND TWO UV202 TRANSCONJUGANTS

| | Restriction fragment sizes (kb) | | | | |
|---|---|---|---|---|---|
| Strain | *Bgl*II | *Eco*RI | *Hin*dIII | *Kpn*I | *Pst*I |
| DP1322 | 7.95 | 3.8<br>3.27 | 5.4 | 2.75 | > 10 |
| Fx21-1[a] | > 10<br>9.4 | 6.2<br>3.9 | 6.8<br>4.1 | > 10<br>4.3 | > 10[b] |
| Fx21-19[c] | > 10 | 3.0 | 5.4 | > 10 | > 10 |
| UV202 | n.t. | n.d. | n.t. | n.t. | n.t. |

a Fx21-1 is a UV202 Tn*5253* transconjugant.

b This band represents a doublette.

c Fx21-19 is a UV202 Tn*5252* transconjugant.

n.d. None detected.

n.t. Not tested.

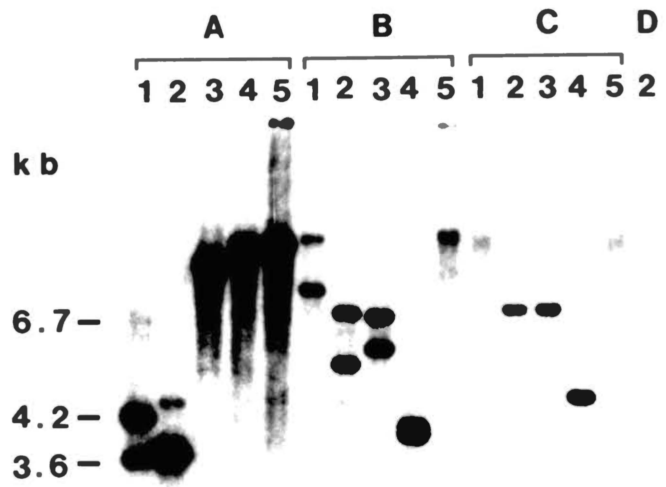


Figure 10. Analysis of the left junction region of two UV202 transconjugants. $^{32}$P-labeled pVJ414 was used as a probe in hybridization with chromosomal DNA from (A) DP1322, (B) Fx21-1, a UV202 transconjugant containing Tn5253, (C) Fx21-19, a UV202 transconjugant containing Tn5252, and (D) *E. faecalis* UV202 digested with (1) *Bgl*II, (2) *Eco*RI, (3) *Hin*dIII, (4) *Kpn*I, and (5) *Pst*I.

TABLE 10

SUMMARY OF RESULTS OF HYBRIDIZATION BETWEEN pVJ414 AND TWO UV202 TRANSCONJUGANTS

| | Restriction fragment sizes (kb) | | | | |
|---|---|---|---|---|---|
| Strain | BglII | EcoRI | HindIII | KpnI | PstI |
| DP1322 | 4.24<br>3.6<br>0.45 | 3.6 | > 10 | > 10 | > 10 |
| Fx21-1[a] | > 10<br>7.5 | 6.7<br>5.4 | 6.6<br>5.8 | 4.1<br>4.0 | > 10[b] |
| Fx21-19[c] | > 10 | 6.9 | 7.0 | 4.7 | > 10 |
| UV202 | n.t. | n.d. | n.t. | n.t. | n.t. |

a Fx21-1 is a UV202 Tn5253 transconjugant.

b This band represents a doublette.

c Fx21-19 is a UV202 Tn5252 transconjugant.

n.d. None detected.

n.t. Not tested.

and left junction probes in the Tn5253 transconjugant revealed one unique fragment size and one fragment of similar size. The finding that each junction probe reacts with a common fragment could be explained by the presence of tandem repeats within the chromosome or a circular form of Tn5253. Only one copy of Tn5252 exists in the UV202 transconjugant, Fx21-19.

# CHAPTER IV

## DISCUSSION

Genomes are usually regarded as somewhat static, changing slowly through evolutionary time by spontaneous mutations created by ultraviolet light or chemical mutagens. However, one must wonder if this slow rate of change can account for the vast diversity among living organisms. In fact, DNA is more dynamic than previously thought. Today it is known that genomes evolve by spontaneously rearranging existing sequences and by acquiring new sequences.

A potent driving force for change within prokaryotic and eukaryotic genomes can by provided by transposable elements or transposons. A transposable element is a discrete segment of DNA with the ability to mobilize and to insert itself at a new location within a replicon independent of sequence homology. Transposable elements not only promote rearrangements of the genome, but, the transposition event itself may cause deletions or inversions of host sequences. For example, transposable elements are thought to play a role in introducing deletions and rearrangements involved in creating the enormous diversity among immunoglobulins.

Some transposable elements mobilize via an RNA

intermediate and reverse transcriptase. As a group, these elements are termed retroposons. These range from retroviruses, which are able to produce an extracellular infectious particle of RNA enclosed in proteins, to sequences which are limited to intracellular transposition with an RNA intermediate (41). The simplest retroposons may be ancestral progenitors to the highly complex retroviruses. Bacteriophage Mu exerts a potent mutagenic effect on its host, _E. coli_, through its ability to integrate virtually anywhere in the host genome (29). Essentially, phage Mu could be described as a transposon disguised as a virus. In light of this, one could ask, are conjugative transposons actually viruses disguised as transposons?

Conjugative transposons are transposable elements with the ability to promote their own DNase resistant transfer between cells independent of any plasmid DNA or phage particle. Recently, conjugative transposons with multiple antibiotic resistance have emerged within the genus _Streptococcus_. Naturally occurring resistance plasmids are relatively rare among pneumococci while multiply resistant conjugative elements are much more prevalent. It is becoming increasingly evident that these conjugative transposons play an important role in the dissemination of antibiotic resistance among this species (7).

Almost all of the streptococcal conjugative

transposons, which range from 16 kb to more than 60 kb in size, contain a homologous tetracycline resistance determinant. Substantial homology between Tn916 and sequences surrounding the tet determinant of several larger elements (greater than 50 kb) has been identified (24). These studies seemed to have provided a clue concerning the evolution of such elements. The identification of a homologous tet determinant has led to the hypothesis that Tn916 represented a prototype conjugative transposon from which all streptococcal conjugative elements were derived (9).

However, the tet region that is homologous to Tn916 within Tn5253, was identified as an independent conjugative transposon, Tn5251. Similarly, the sequences homologous to Tn916 within Tn3701 have been identified as an independent transposon termed Tn3703 (23). Together, these findings seem to argue against the proposed Tn916 prototype model. The aim of this study was to gain insight into the possible role of a Tn916-like element as an ancestor in the evolution of these large streptococcal conjugative transposons through study of the structural and functional features of Tn5253 devoid of the Tn916-like sequences.

If Tn5253 is a derivative of a Tn916-like ancestor and dependent on the Tn916-like sequences in conjugative transfer, then Tn5253 devoid of Tn5251 should be transfer deficient. However, if the Tn916-like sequences were

added on to a preexisting element, then Tn5253 devoid of Tn5251 should retain its conjugative and other properties.

The recombinant plasmid pVJ403Δ*tet* was used as a tool to induce the in vivo deletion of the Tn5251 sequences from Tn5253. A strain carrying this deletion was created as described and the predicted structure was confirmed by Southern hybridization. A strain of pneumococcus harboring this deletion within Tn5253 was used as a donor in filter-mating experiments to determine whether these remaining sequences were capable of conjugative transfer. The sequences beyond Tn5251 within Tn5253 were found to be transfer proficient with conjugative properties similar to those of the parental element, Tn5253. This independent conjugative transposon was termed Tn5252. Conjugative transfer of Tn5252 to pneumococcal recipient cells was at a frequency comparable to that of the parental element, Tn5253 ($10^{-7}$ to $10^{-6}$ transconjugants per donor). In addition, analysis of Tn5252 transconjugants by Southern hybridization revealed that Tn5253 and Tn5252 integrated at the same target site within the pneumococcal chromosome.

The interspecific transfer of Tn5252 was confirmed by using *E. faecalis* recipients in filter mating experiments. Unlike pneumococcus, *E. faecalis* is not known to transform naturally. Thus, transfer of Tn5252

to E. faecalis was not expected to occur via transformation but only through a conjugative process. The frequency of transfer of Tn5252 to E. faecalis was comparable to that of the parental element with $10^{-5}$ to $10^{-6}$ transconjugants per donor. Analysis of several transconjugants revealed that Tn5252 inserted into more than one site in the E. faecalis chromosome. Analysis of Tn5253 transconjugants showed that Tn5253 inserted into the E. faecalis chromosome at a different site than Tn5252.

No homology was detected between the E. faecalis chromosome and the target for Tn5252 in S. pneumoniae Rx1. Although imperfect homology may exist between the E. faecalis chromosome and the Rx1 target probe, such homology would not be detected with the level of stringency used in this hybridization. Hybridization performed under less stringent conditions may reveal some degree of homology. However, integration of Tn5252 into the E. faecalis chromosome appears to be a homology independent process. In addition, Tn5252 was observed to integrate into more than one target site within the E. faecalis chromosome. Further, integration of Tn5252 into the chromosome of recombination deficient E. faecalis UV202 attests to the independence of Tn5252 from a competent host recombination system. Together, these findings strongly confirmed that Tn5252 was also a transposable element.

Tn5252 retained conjugative transposition properties similar to those of Tn5253 and different from those of Tn5251. This indicates that Tn5251 probably does not play a mechanistic role in conjugative transfer of Tn5253. Therefore, structurally, Tn5253 is a composite element consisting of two independent conjugative transposons, Tn5251 and Tn5252. From the data generated in this study, it is likely that a Tn5252-like element is the prototype for this and other larger conjugative transposons rather than the internal Tn916-like element. This observation agrees with the findings of Le Bouguenec, et al. (24). These results seem to indicate that there may be two classes of conjugative transposons in streptococci, the smaller elements similar in structure and function to Tn916 and larger elements similar in structure and function to Tn5252.

Interestingly, while Tn5252 and Tn5253 insert into a preferred target site in the pneumococcal chromosome, Tn5252 inserted into more than one site after transfer to *E. faecalis* and the parental element appeared to insert into yet another site in the *E. faecalis* chromosome. This parallels the findings of Inamine, et al. (20). By Southern hybridization analysis, they observed that Tn3951, a conjugative transposon very similar to Tn5253, had a preferred target site for insertion into the *S. agalactiae* chromosome, but interestingly, had multiple target sites in *E. faecalis*.

Although E. faecalis UV202 had no detectable homology to the target site for Tn5253 in Rx1, all of the E. faecalis transconjugants analyzed containing either Tn5252 or Tn5253 showed weak homology to the Rx1 target probe. The fragment sizes reacting with the target probe varied in size and paralleled the observed differences in target selection between isolates. This seems to indicate that the observed homology is located at the termini rather than within the element itself. This observation suggests that a small amount of DNA from the donor target region may have been transferred into the recipient.

Although the mobilization of donor DNA does not appear to be extensive (perhaps less than 100 bases), it is intriguing. Caparon and Scott have observed a similar process occurring in the transposition of Tn916 (6). They have identified an intracellular, covalently closed, circular, double stranded form of Tn916 believed to act as an intermediate in transposition. They have obtained the DNA sequence of the fusion fragment created by the joining of the termini of Tn916 in formation of this circle. In addition, they have obtained the DNA sequence of a Tn916 target on a plasmid replicon before insertion and after excision of the element. By comparing the observed sequences, they determined that upon excision of the element, Tn916 mobilizes five bases of the target DNA. According to their model, these five bases are then

transferred into the recipient genome upon integration of Tn916. Although this may not explain the nature of Tn5252 itself, future investigation of this observation may give insight on the mechanism of transfer of this and related elements.

The findings in this study showed that Tn5253 was composed of two independent conjugative transposons with the smaller Tn5251 inside of the larger Tn5252 element. Spontaneous separation of these two elements during conjugative transfer has not been observed. However, the Tn5251 sequences excised when removed from the context of the larger element and subcloned onto an *E. coli* plasmid vector. This spontaneous loss of Tn5251 from pVJ403 led to the identification of this transposon. Perhaps, by some yet unknown mechanism, sequences surrounding Tn5251 as it exists in Tn5253 regulated its independent movement and this "repression" was disrupted when Tn5251 was subcloned from Tn5253.

The mechanisms involved in conjugal transfer of streptococcal conjugative elements is unknown. Several models have been proposed (6,8,9,16,20) which involve transfer of a non-replicative, circular intermediate. Strong evidence to support this model has been provided with the isolation and characterization of an excised circular form of Tn916 as mentioned above (6).

Hybridization analysis of the termini of Tn5253 in UV202 has produced some interesting results to support

this model of a covalently closed circular intermediate form of Tn5253. In both *E. faecalis* UV202 transconjugants analyzed containing Tn5253, two copies of the element were identified. Hybridization with probes from the right junction (Figure 9) and left junction (Figure 10) regions produced one restriction fragment of a unique size and a second restriction fragment of similar size (Table 9 and Table 10).

A common restriction fragment reacting with both junctions of Tn5253 could have been produced by the fusion of the left and right termini and circularization of Tn5253. If this second copy of Tn5253 in UV202 is a circular form of the element, continued study of this observation may provide evidence for the mechanisms involved in conjugal transfer of this element. Interestingly, only one copy of Tn5252 was detected in the *E. faecalis* UV202 transconjugants. Failure to detect a second copy of Tn5252 in UV202 transconjugants may have been related to the absence of the Tn5251 sequences in this element. It is also possible that a second circular copy of Tn5252 was present but was not detectable at the level of stringency used in these hybridizations. Or, perhaps sequences within Tn5251 were somehow involved in the formation of this proposed circular form in *E. faecalis* UV202.

Based on restriction analysis, Southern hybridization, and similarities in the functional

features, Tn5251 appears to be related to Tn1545 and Tn916 (1,12,13,24,39). The terminal nucleotide sequences of Tn1545 and Tn916 are almost identical for at least 250 bp (5,8). Further, in contrast to other classes of transposable elements, Tn1545 and Tn916 do not produce a duplication of the target sequences upon insertion. DNA sequence analysis of the termini and target site of Tn5251 should be investigated for further comparison of this element to the Tn916 class of conjugative transposons and to examine the extent of structural similarities between these related elements.

During the excision of Tn5251 from the recombinant plasmid pVJ403, the sequences that flanked the transposon were spliced together to create a fusion fragment. The product of this excision, pVJ403Δ tet, contained the target sequences for insertion of Tn5251 into Tn5253. By comparing the restriction maps of pVJ403 and pVJ403Δ tet, the fusion site was mapped to within a 0.8 kb fusion fragment flanked by HindIII and BamHI sites. This 0.8 kb fusion fragment has been subcloned from pVJ403Δ tet onto the plasmid vector, pUC8, to generate the recombinant plasmid designated pAT101. Similarly, by comparing the restriction maps of pVJ403 and pVJ403Δ tet, restriction fragments containing the left or right termini of Tn5251 have been identified and localized. A restriction fragment containing the left junction region of Tn5251 in Tn5253 has been subcloned from pVJ403 onto the plasmid

vector, pUC8, to generate the recombinant plasmid pAT103. A recombinant plasmid containing passenger DNA from the right junction region has not been obtained.

DNA sequence analysis of the passenger DNA of these recombinant plasmids has been initiated. However, due to time constraints, these studies have not been completed. Efforts to attain complete sequence analysis of the target site and junction regions of Tn5251, as it exists in Tn5253, will continue in this laboratory.

Based on Southern hybridization analysis and differences in function features, Tn5253 and Tn5252 are clearly different from the Tn916-like elements (1,12,13, 24,29,35). Comparison of the nucleotide sequence of the target site and junction regions of Tn5252 and Tn5253 may reveal the extent of structural difference. In addition, sequence analysis of the termini of Tn5252 and Tn5253 in *E. faecalis* UV202 transconjugants may reveal the nature and extent of mobilization of donor sequences into the recipient cells during conjugative transfer. This may also give insight into the processes involved in the transfer of this class of transposable elements. Further, Tn5252 is an independent conjugative transposon with functional properties similar to those of Tn5253. Localization of transfer related genes within this 47 kb element may shed light on the mechanisms of newly emerging multiple antibiotic resistance in streptococci.

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