STRUCTURAL AND FUNCTIONAL ANALYSIS OF A REGULATORY REGION IN THE STREPTOCOCCAL CONJUGATIVE TRANSPOSON, Tn*5252*

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Submitted to the Faculty of
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Oklahoma State University
in partial fulfillment of the
requirements for
the Degree of
DOCTOR OF PHILOSOPHY
July, 1997

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Dedication

Dedicated with deepest affection to my parents, who gave me life and inspired me to be the best I can be.

ACKNOWLEDGMENTS

I am deeply indebted to my thesis adviser Dr. Moses Vijayakumar who taught me "to do science the right way". I was fortunate to have him as a scientific mentor who with his strong passion for science has left a profound influence on my style of thinking about molecular genetics.

I greatly appreciate the suggestions and guidance of my scientific committee members during the development of thesis research.

It is as always a pleasure to thank colleagues of whom the following deserve special mention: Ali Kilic for his enthusiasm and support from the beginning of this project, Francisco Alarcon-Chaidez for his expert advice on taming the computer, Janardhan Sampath for always being there, and Ursula Munoz-Najar for all the interesting conversations and for sharing the female point of view.

I also would like to thank Tara Bennet and Audrey Sheridan for cleaning up my mess, and Sandra Peterson, for seeing to my autoclaving needs.

Deepak Girdhar gave me the encouragement and gentle prodding that kept me from getting terminally sidetracked. To him I owe my thanks.

Finally, I would like to thank my family and friends for their support and patience with my constant preoccupation with science and all those who have made this a pleasant memory.

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CHAPTER I

INTRODUCTION

Streptococcus pneumoniae is the primary etiological agent of pneumonia among human patients. In spite of the widespread use of several antibiotics to combat infections due to *S. pneumoniae*, endogenous plasmids carrying antibiotic resistance genes have not been isolated from this group of bacteria. However, the antibiotic resistance genes have been found to be associated within the genome on a class of novel, mobile, genomically inserted elements, called conjugative transposons. Conjugative transposons appear to be solely responsible for the dissemination of antibiotic resistance markers in *S. pneumoniae.* Our lab has been channeling its efforts towards studying one such element called Tn*5253.*

Tn*5253*, formerly called the Ω (cat tet) element was originally detected as a heterologous insertion in the chromosome of the plasmid-free clinical isolate *S. pneumoniae* BM6001. By inserting an *Escherichia coli* vector plasmid (pVA891) which is incapable of autonomous replication in streptococci at many sites specifically within Tn*5253*, Vijayakumar et al. (82) were able to clone and recover parts of the element in *E. coli*. Physical analysis of the passenger DNAs from these plasmids made it possible to construct a detailed restriction map of this 65.5 kb element, its junction and target regions in the pneumococcal chromosome. The transposition behavior of a DNA segment carrying tetracycline resistance when separated from the context of the surrounding DNA led to the identification of Tn*5253* as a composite structure of two independent conjugative transposons: an 18 kb element Tn*5251* (Tc^r) inserted in the central region of another, Tn*5252* (47 kb, Cm^r). Studies from our lab have shown that Tn*5251* is structurally and functionally similar to Tn*916*.

The structure and mechanism of transposition of Tn*916* and Tn*1545*, the smaller class of conjugative transposons, have been the subject of investigation by several labs. On the other hand, our knowledge of the larger conjugative transposons is very limited. In spite of apparent functional similarities between the two classes of conjugative transposons, crucial differences were also observed, in particular with reference to their target specificity. While the smaller elements insert at more than one genomic site, the larger elements such as Tn*5252* seemed to prefer to insert at a unique site in pneumococcus. Studies in our lab support the notion that the larger conjugative transposons are very different from the smaller Tn*916* class of elements and belong to a distinct class of mobile elements that carry site-specific integration-excision functions.

Recent studies in our lab have revealed several transfer related regions within Tn*5252*. Heterologous insertions at specific regions in the transposon led to the loss of transfer functions, indicating that probably these regions were involved in the conjugal transposition. The transfer related regions seemed to be clustered towards the termini of the element. Several regions in the central portion of the element were found to be non-essential for the conjugal transposition of the transposon. These data indicated that this segment of DNA could acquire insertions such as Tn*5251* or the *cat* determinant without affecting transposition of Tn*5252*. Since nothing is known regarding the functional aspects for these regions, we decided to sequence a few kilobases of DNA from the left terminus spanning one of the regions which was involved in the conjugal transfer of the element (1, 37, 38).

Specific Aims of the Proposed Research Project

1. To obtain the nucleotide sequence of about 4.5 kb of DNA flanked by *Xba*I sites between coordinates 3.5 to 8.0 at the left terminus of Tn*5252*.
2. To identify the open reading frames present within this sequenced region.

3. To mutagenize the ORFs and determine their roles in conjugal transposition of Tn*5252*.

4. If an ORF is found to be necessary for the transfer of the element, to isolate the gene product in large quantities for structural and functional studies.

The genetic and functional properties of this element would be characterized using recombinant DNA techniques, transformation, filter mating experiments, protein purification, and DNA binding assays.

Significance of the Study

Earlier studies in our lab have indicated that transfer related regions are clustered more towards the termini of the element. Sequence information has shown the presence of an integrase gene in the left most region of the element. Mutation of this gene led to the loss of conjugal transfer of the element. An insertional mutation of a region a few kilobases upstream of the integrase region also seemed to inhibit conjugal transfer of the element. Therefore it seemed that the left terminus was important for the intercellular movement of Tn*5252*. Tn*5252* , as discussed earlier, belongs to a distinct class of conjugative transposons that are very different from the Tn*916* class of elements which are the focus of study in several research labs. In light of the emergence of multiple antibiotic resistance in pneumococci which is solely due to the presence of conjugative transposons, it is important to understand the mechanics of movement of these elements between cells. Our lab is the only one involved in the study of Tn*5252* like elements. As these types of elements seem to have functionally replaced plasmids in pneumococci, and the presence of multiple antibiotic resistance determinants are commonly found on these, a detailed understanding of these novel elements becomes important in terms of basic biology as well as clinical medicine.

Results so far have already yielded clues concerning possible mechanisms for the transfer of the conjugative element, Tn*5252*. However, a lot still needs to be done. Using

the information that will be obtained in this study, a plausible mechanism of transposition of Tn*5252* will be proposed. Answers to these questions would enlarge our understanding of the mechanism of gene transfer among prokaryotes at large. This would also enable us to devise ways to curtail their spread.

CHAPTER II

BACKGROUND

The Streptococcus

Several species of gram-positive cocci have their main habitat in the upper respiratory tract of man. They include the streptococci and staphylococci, and within each of these groups there are commensals, pathogens and potential pathogens, the last of which affect tissues with lowered resistance. The streptococci belong to the family Streptococcaceae and are spherical or oval cells arranged in chains of varying length, non-motile, non-sporing and may be capsulate. The majority are facultative anaerobes, but there are species that are anaerobic. Most streptococcal infections like pharyngitis, scarlet fever, skin infections, erysipelas, impetigo, rheumatic fever, necrotizing fasciitis, and acute glomerulonephritis are caused by the group A *Streptococcus pyogenes*. *Streptococcus agalactiae* is associated with a number of human infections, including urinary tract infections, pneumonia, abscesses, wound infections, meningitis, endocarditis, neonatal infections, and septic abortions. Viridans streptococci play a major role in the causation of dental carries, periodontal infections and subacute bacterial endocarditis. *Enterococcus feacalis* is an opportunistic pathogen when it gains access to the urinary tract. The pneumococci, *S. pneumoniae*, are the most common primary pathogens in lobar, lobular and broncho-pneumonia, and bronchiolitis. This organism is also the most common cause of sinusitis, acute bacterial otitis media and conjunctivitis beyond early childhood. The pneumococci are, therefore, major contributors to a vast amount of morbidity and economic loss since respiratory infections are responsible for 30 to 40% of the illnesses requiring medical attention and of sickness absenteeism in schools and industries. They are

particularly frequent and severe in the extremes of life. Therefore the streptococci constitute a virulent group of bacteria associated with several human infections.

Drug Resistance in Pneumococci

Members of the bacterial genus *Streptococcus* are responsible for a wide variety of human illnesses. Antibiotic resistance is common in these organisms and frequently involves nonplasmid elements.

In the last few decades, pneumococci have acquired resistance to a number of antibiotics (71). Resistance to sulfonamide appeared in clinical strains of pneumococcus as early as 1939 followed by tetracycline and penicillin resistance. In the 1960s erythromycin resistance appeared. In the 1970s multiple antibiotic resistant strains of clinical *S. pneumoniae* of many serotypes suddenly emerged (20, 23, 36). Most of these isolates were resistant to chloramphenicol, erythromycin or kanamycin in addition to tetracycline (36). The chloramphenicol resistance is acquired by the presence of the *cat* gene which encodes a chloramphenicol acetyl transferase which detoxifies chloramphenicol (50). The *erm* gene product confers resistance to the macrolide, lincosamide and streptogramin B (MLS^r) group of antibiotics, including erythromycin. MLS resistance is due to methylation of the 23 S subunit of ribosomal RNA. Kanamycin resistance in pneumococci is due to aminoglycoside 3'-phosphotransferase, which is the product of the *aph*A gene. Tetracycline resistance is due to the *tet*M gene, whose product binds to the 30 S subunit of the ribosome and makes it insensitive to tetracycline inhibition (27).

Source of Antibiotic Resistance

During the latter part of the 1970s, when the emergence of multiple drug resistant strains of *S. pneumoniae* was becoming a clinical concern, investigators in several labs

were unable to identify related plasmids (10, 20, 60, 69). Even though pneumococci can accept and maintain a variety of plasmids from other streptococci, for reasons that are not readily apparent, endogenous extrachromosomal elements have only been rarely observed in this species. Shoemaker et al (69) provided the first evidence for the chromosomal location of the drug resistance determinants. They observed that *cat* transforming activity, which is relatively insensitive to shearing of donor DNA, cosedimented with chromosomal DNA markers both in unsheared lysates and after shearing them to much smaller DNA sizes (69). These chromosomal elements were able to transfer within and between different species of streptococci on nitrocellulose filters by a process requiring cell-to-cell contact (16, 72) and which was DNase resistant, eliminating the possibility of transformation or generalized transduction (29). Horodniceanu et al. (33) reported similar observations in *S. pyogenes*, *S.agalactiae*, and streptococcal Lancefield groups F and G. Some of these systems were found to involve large segments (60 kb) of chromosomal DNA containing two or more resistant determinants and eventually proved to be relatively complex conjugative transposons. However, Courvalin and associates found one pneumococcal element, Tn*1545* to be only 25 kb (18, 19).

Conjugative Transposons

Antibiotic resistance among clinically important gram-positive bacteria is often determined by the presence of mobile genetic elements carrying resistance genes. These transposable elements were named conjugative transposons (14, 26) due to the fact that these elements confer on their gram-positive hosts the ability to act as conjugative donors. During conjugative transposition the element is transferred to a new location, most of the times in the genome of the recipient strain.

Conjugative Transposons are Unique

Conjugative transposons are transposon-like in the sense they excise from and integrate into DNA, but they appear to have a fundamentally different mechanism of excision and integration from that of the well-studied transposons found commonly in *E.coli*, such as Tn*5* and Tn*10*. For example, conjugative transposons have a covalently closed circular transposition intermediate and do not duplicate the target site when they integrate into the recipient genome (15, 58, 65, 66). They are also plasmid-like in some properties, in that they have a circular transfer intermediate that is transferred by conjugation, but unlike plasmids, the circular intermediate of a conjugative transposon does not replicate, at least in systems so far investigated (58, 65). Conjugative transposons are also phage-like in that their transposition resembles excision and integration functions of temperate bacteriophages, which have a circular intermediate. However, in contrast to the lambdoid phages, conjugative transposons do not form viral particles, and they are not transferred by transduction.

Classification of Conjugative Transposons

Conjugative transposons were first discovered from streptococcal strains associated with human disease in the late 1970s by two different laboratories (25, 26, 68). Tn*916*, found in *Enterococcus faecalis* by Clewell's group (25,26) and the conjugative transposon found in *S. pneumoniae* originally called the Ω*cat/tet* element and now called Tn*5253* (4, 68) were the first two transposons identified. Since then, a number of conjugative transposons have been isolated and characterized.

Substantial progress has been made over the last decade toward the genetic and molecular characterization of these mobile elements which range in size from 16 to 70 kb in size. On the basis of size, conjugative transposons could be classified into two types, one

ranging in size from 16 to 25 kb and the other being over forty kb. Tn*916* was the first conjugative transposon identified representing the first category. It is 18.5 kb in size and harbors a tetracycline resistance determinant. This element was isolated from *E. faecalis*. Several conjugative transposons resembling Tn*916* have since then been identified. Therefore this family of closely-related transposons are called the Tn*916*-like elements. This family includes, Tn*1545* from *S. pneumoniae*; Tn*918*, Tn*920*, Tn*925*, and Tn*3702* from *E. faecalis*; Tn*5031*, Tn*5032*, and Tn*5033* from *E. faecium*; Tn*919* from *S. sanguis*; and Tn*5251* from *S. pneumoniae*. Many carry a combination of drug resistance determinants, however, most of the elements appear to carry tetracycline resistance of the *tet*M type (8, 9) or a closely related gene for tetracycline resistance that expresses in both gram-positive as well as gram-negative bacteria. The Tn*916*-like elements are also very simple in that they are observed to transpose as a unit. The larger transposons, typified by Tn*5253*, are over sixty kb in size, much more complex and may be composed of several transposable elements. The best studied of these are Tn*5253* from *S. pneumoniae*, Tn*3701* from *S. pyogenes*, and Tn*5276* found in a *Lactococcus lactis* strain.

Based on structural properties, four distinct types of conjugative transposons have been identified (15). Of these the ones found in *Bacteroides* seem to be very different from the gram-positive elements (61). The three types of gram-positive elements are the Tn*916*-like elements, the Tn*5252* class elements and the lactococcal elements. Despite the absence of any striking homology at the DNA sequence level to each other, the functional behavior of these three types displays a basic unifying theme. Representative transposons from each class studied with reference to their molecular structure have been found to carry genes specifying recombination functions at one end of the element (38, 54, 57). The target regions for integration of these elements are usually rich in A/T base pairs (15). However, when it comes to target specificity, there is considerable difference between the Tn*916* class and the other two classes of transposons. Tn*5276* (57) and Tn*5252* (84) prefer to insert at a unique site in their host genomes while the Tn*916*/Tn*1545* class of

elements integrate at several sites with a decided preference for certain spots indicating degenerate specificity (64). The relatively small sizes of Tn*916* and Tn*1545* have facilitated their characterization to a great extent. Although, the entire DNA sequence of Tn*916* has been determined, a clear picture of the mechanism of conjugative transposition has not yet emerged.

Conjugative Transposons of Gram-negative Bacteria

Conjugative transposons are not unique to the gram-positive bacteria; they have also been reported in gram-negative bacteria. *Neisseria gonorrhoea* and *Kingella* spp., both gram-negative bacteria, have been found to contain Tn*916* and its relatives (39). This is not surprising at all, since Tn*916* can transfer to gram-negative recipients with good efficiency. A distinct group of transposons, unrelated to the Tn*916* family, have been found in the gram-negative anaerobe, *Bacteroides* spp (61). Two groups of gram-negative bacteria in which conjugative transposons have still not been reported , are the *E. coli* and *Pseudomonas* spp.

The Tn*916*-Tn*1545* Family

Tn*916*, an 18 kb element that encodes resistance to tetracycline, is the most studied transposon of the gram-positive transposable elements. It was found on the chromosome of the hemolytic multidrug-resistant *E. faecalis* DS16 (25). Tn*916* was shown to be able to insert into various sites on the highly conjugative resident plasmid, pAD1 (13). It was also shown to transfer intercellularly from cells lacking the plasmid and was found to insert at multiple sites on the recipient chromosome (26).

Transfer intercellularly occurs by a *rec* independent process, and the transconjugants (recipients that have acquired the transposon) were found to have widely

different donor potentials for subsequent transfers. Transfer frequencies range from $<10^{-9}$ to $>10^{-4}$. Interestingly, half the members of the transconjugants had more than one copy of the transposon, sometimes as many as six copies per cell (26). Tn*916* and related elements do not generate direct repeats at their junctions (11). Excision of the element was precise and insertion of the intermediate at its target site did not lead to target duplication (12). Physical evidence reported by Scott et al (65) strongly supported the existence of a circular intermediate. Circular intermediate formation was also reported in a Tn*916*-like element designated Tn*5381*. In the presence of a subinhibitory concentration of tetracycline, an increase in conjugal transfer frequency and circular intermediate formation was also observed (59). Other than transferring to gram-negatives, Tn*916* has also been observed to transfer to soil populations of *Streptomyces* spp. (51).

Tn*1545* (25 kb) is larger than Tn*916* and carries kanamycin and erythromycin resistance genes in addition to *tet*M. Its ends and most of its interior are almost identical to corresponding regions of Tn*916* (11, 17). Tn*1545* also does not generate target dulpications at the site of insertion (11). Two genes whose products may be involved in the excision and integration of the element similar to those of the Xis and Int proteins of bacteriophage lambda were also identified (55). Tn*916*-Tn*1545* have a remarkably broad host range. Members of this family have been introduced into, or occur naturally in, at least 52 different species of bacteria from 24 genera (17).

COMPOSITE TRANSPOSONS

Structure of Tn*3701*

Tn*3701* is one of the best studied composite transposons. It was discovered in *S. pyogenes* A54 and encodes resistance to chloramphenicol, erythromycin and tetracycline (44). Within Tn*3701* is a region homologous to Tn*916* that has an insertion carrying

resistance to erythromycin in addition to tetracycline. This region, designated Tn*3703*, has been shown to transpose from a plasmid to the chromosome, but does not undergo conjugative transfer (32, 44). The reason for this lack of intercellular transfer function could be due to the insertion of the *erm* segment in a gene required for conjugation. The *erm* marker and adjacent DNA have been found to spontaneously cure, indicating that *erm* may be on a separate element which is nonconjugative (44).

Tn*3951*

A composite conjugative transposon called Tn*3951* was identified in *S. agalactiae* B109. This transposon is 67 kb in size and confers resistance to chloramphenicol, erythromycin and tetracycline (35). Tn*3951* was able to conjugatively transfer to *E. faecalis* recipients (70). Upon introduction of the hemolysin plasmid pAD1 into the transconjugants, Smith et al (70) observed Tn*3951* to have transposed to the plasmid generating a nonhemolytic strain. Like Tn*3701*, the *erm* and *tet* determinants of Tn*3951* showed extensive homology to Tn*916*. Both these markers were carried on a 22 kb *Eco*RI fragment and were able to express in *E. coli* DB11 strain. Following conjugal transfer of the element to pneumococci, it was observed that Tn*3951* was site-specific, a feature shared by the larger elements (59).

The Lactococcal Element

Tn*5276* is a 70 kb complex conjugative transposon identified in *Lactococcus lactis* (57). Tn*5276* seems to be unrelated to Tn*3701* or Tn*5253*. It carries a gene involved in sucrose metabolism and a gene encoding resistance for nisin, a bacteriocin used by the food industry to prevent growth of undesirable bacteria.

Organization of Tn*5253*

Studies in our lab have focused on the investigation of Tn*5253*, belonging to the large composite transposons family. Tn*5253*, formerly called the Ω*cat/tet* element, was originally detected in a plasmid-free clinical strain of *S. pneumoniae* BM6001 as a heterologous insertion in the chromosome (10, 20). Tn*5253* is a 65.5 kb self-transmissible element that encodes resistance to tetracycline and chloramphenicol (Figure 1). Conjugal transfer frequency of the element to other streptococci ranged between 10^{-7} to 10^{-6} per donor cell (71). The *cat* and *tet* determinants were found to be linked together, based on transformation criteria. Furthermore, they comigrated with the chromosomal markers in velocity sedimentation and buoyancy gradients, eliminating the possibility of plasmids (71).

Identification of Tn*5251*

Vijayakumar et al (82, 85) cloned the entire element in fragments in *E. coli* and generated its restriction endonuclease map. This allowed them to localize the drug resistance determinants and to identify its junction and target regions on the chromosome of pneumococci. The transposition behavior of a DNA segment carrying the tetracycline resistance determinant when separated from the context of the surrounding DNA led to the identification of Tn*5253* as a composite transposon consisting of two independent conjugative elements (4). Interestingly, they identified a region similar to Tn*916* and they named it Tn*5251*. Hybridization studies done in this laboratory as well as others (45) suggested that Tn*5251* was closely related to the Tn*916*, Tn*1545*, and Tn*3703* class of transposons and structurally distinct from the Tn*5252* class of elements. The internal Tn*916*-like region is capable of independent conjugative transposition in that it displayed conjugal transfer properties when removed from the larger element, Tn*5253*. It is 18 kb in

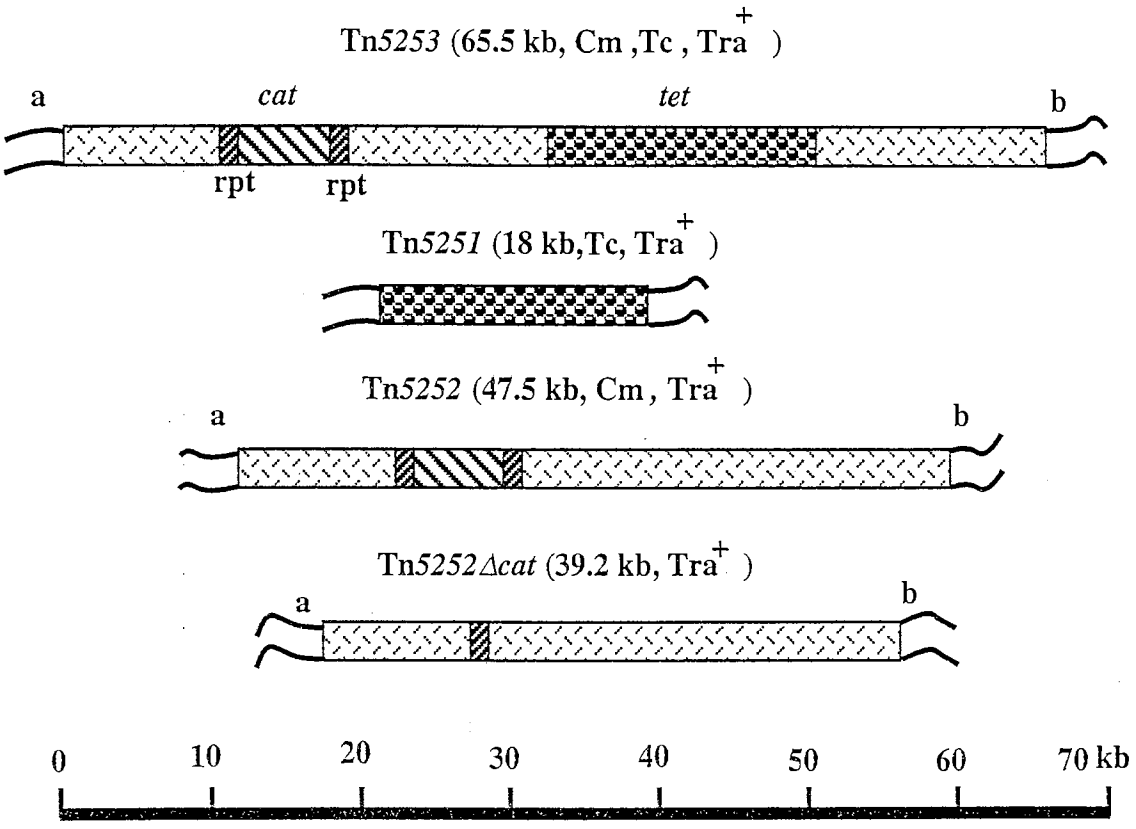


Figure 1. Composite nature of Tn*5253* and its derivatives. a and b are arbitrarily chosen left and right chromosomal regions flanking the transposon. Separation of Tn*5251* from the larger element results in Tn*5252*. The *cat* region located in the left part of the element is flanked by direct repeats of 1.7 kb and often spontaneously cures. The transfer functions of the remaining Tn*5252* Δ*cat* are not impaired (38).

size and carries the *tet*M determinant. Deletion of Tn*5251* from the context of the surrounding DNA led to the identification of Tn*5252*.

Structure of Tn*5252*

Tn*5252* is made up of sequences beyond Tn*5251* within Tn*5253*, constituting 47.5 kb of DNA. Tn*5252* bears the *cat* determinant and is capable of conjugative transposition. It seemed to insert at more than one site following conjugal transfer in *E. faecalis*. The transfer of this element to *E. faecalis* indicated that this transfer was not dependent on host recombination functions. Unlike Tn*5251*, Tn*5252* seemed to prefer to insert at a unique target site in pneumococci, a behavior similar to that of the parental element Tn*5253*. This indicates that Tn*5251* did not play a mechanistic part in the conjugation of the larger element.

Even though Tn*5251* and Tn*5252* were both capable of autonomous conjugal transfer, separation of these elements has not been observed when they are associated as Tn*5253*. The inability of Tn*5251* to move independently from within Tn*5253* may be due to the flanking sequences in Tn*5252*. Moreover, the fact that the two elements choose different target sites following conjugal transfer indicates that they could have different modes of transfer. The lack of restriction of Tn*5253* while simultaneously entering plasmids were being restricted by the *Dpn*II system suggests the possibility that the mechanism of transfer of the larger elements may be quite different from that of Tn*916* (29). All these results led to the conclusion that smaller elements such as Tn*5251* were added at a later date to larger ones like Tn*5252* to create complex transposons such as Tn*5253*. Therefore one cannot consider Tn*916*-like transposons to be progenitors of the larger elements. Results from our laboratory suggest that a Tn*5252*-like element should be considered a prototype of the composite conjugative elements such as Tn*5253* in agreement with earlier observations (45).

The *cat* region is flanked by direct repeat sequences that also appear in the wild-type pneumococcal genome. These are probably related to the frequent spontaneous curing of the *cat* region and to its ability to separate from *tet* during transformation, indicating that these could be IS-like elements or their remains (85). The presence of an internal transposon and the IS-like elements in Tn*5253* reflects the complexity of the larger elements. The propensity for the autoaccumulation of various genetic units into prototype elements such as Tn*5252* could be due to the presence of integrons. Integrons are DNA elements that encode a site-specific recombination system capable of acquiring genes at a specific site (77).

It has been shown that for the insertion of Tn*5252* into its host genome, a 72 bp segment at the left end of the transposon and an identical copy of the target sequence on the chromosome of the host are required for the homology necessary for site-specific target recognition and insertion (3). The functional map of Tn*5252* also has been established by creating a series of defined deletion and insertion mutations within Tn*5252*. Interruptions at several regions were found to affect the conjugal transposition functions of the element indicating the location of genes possibly involved in the transfer process. Interestingly, insertions in the middle of the element did not affect transfer, which was not surprising, considering that this segment of DNA seemed to be where other mobile elements such as Tn*5251* and the *cat* segment often integrate (38).

This work primarily focuses on a region towards the left end of the transposon Tn*5252* that is involved with transfer functions. The results are expected to shed light on the possible transfer mechanism of the element.

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains

Rx1, a non-encapsulated strain used in this study, is our laboratory strain that is equivalent to the wild type *S. pneumoniae*. The remaining pneumococcal strains are all derivatives of this strain. SP1000 is an Rx1 derivative with a point mutation (*str-1*) conferring streptomycin resistance, and carrying the transposon, Tn*5252*. SP1254 is a derivative of SP1000, in which pVA891 is inserted within Tn*5252* at a locus not involved in transfer (38). The pneumococcal strains used in this study are listed in Table I.

Streptococcus pyogenes strains used for conjugation experiments in this study were ATCC21547 and ATCC19615. The latter was kindly provided by the Stillwater Medical Center.

Plasmids

The generation and preparation of recombinant plasmids were done in *E. coli* strains listed in Table II. Vector plasmids used in this study are pVA891, pLS1, pMAL-p2 and pET-30a-c(+). pVA891 (47) is an *E. coli* plasmid carrying *cat* and *erm* resistance, of which only the *erm* is expressed in pneumococcus. The broad-host-range plasmid pLS1 (76) is a derivative of pMV158 isolated from *Streptococcus agalactiae* (9). Both plasmids can autonomously replicate and express tetracycline resistance in *S. pneumoniae* (63), *Bacillus subtilis* (22) and the recombination-proficient *E. coli* (40). pMAL-2 vectors and

TABLE I

STREPTOCOCCAL STRAINS

STRAIN	GENOTYPE	ORIGIN/REFERENCE
S. pneumoniae		
Rx1	*hex*	67
DP1002	*nov-1*	30
DP1004	*str-1*	30
DP1617	*hex str-1 ery-2 nov-1 fus sulf-d stg*	71
SP1000	*str-1 fus* Tn*5252* (*cat*)	4
SP1254	*str-1 fus* Tn*5252* (*cat*ΩEm) Tra^+	37
SP1261	*str-1 fus* Tn*5252* (*cat*ΩEm) Tra^-	This work
S. pyogenes		
ATCC19615	*opt*	Stillwater Med.Center
ATCC21547	*opt*	ATCC

nov-1 Chromosomal point mutation conferring resistance to novobiocin.

fus Chromosomal point mutation conferring resistance to fusidic acid.

str-1 Chromosomal point mutation conferring resistance to streptomycin.

cat Chloramphenicol acetyl transferase.

opt Optochin.

TABLE II

E. coli STRAINS

STRAIN	GENOTYPE/PHENOTYPE
DH5α	F^-, recA1, $lacZ^+$
JM109	F', recA1, $lacZ^+$
C600	F^-, lacY1
BL21 (λDE3)	F^- *omp* T $r_B^- m_B^-$

pET-30a-c(+) vector plasmids were purchased from New England Biolabs (NEB) and Novagen respectively. These and other plasmids used are listed in Table III.

Enzymes and Chemicals

Restriction endonucleases, DNA molecular weight standards, and most of the commonly used modifying enzymes were purchased from one of the following suppliers: Bethesda Research Laboratories (BRL), Promega Biotech, New England Biolabs (NEB), and United States Biochemical Company (USBC). Sequenase Version 2.0 T7 DNA Polymerase and Deep Vent DNA Polymerase were purchased from USBC and NEB respectively. DNase I, RNase I, antibiotics, and bovine serum albumin (fraction V) were purchased from Sigma Chemical Company. Media and agar for bacterial growth were purchased from Difco and USBC. Agarose for horizontal gel electrophoresis was purchased from Fisher. For electroelutions, molecular biology grade ultrapure agarose from Bio-Rad Laboratories was used. $\{\alpha\text{-}^{32}P\}$-CTP, $\{\alpha\text{-}^{35}S\}$-ATP, $\{^{35}S\}$-Methionine, $\{^{3}H\}$-Leucine, and $\{^{14}C\}$-methylated proteins were purchased from New England Nuclear Research Products (NEN). Primers for sequencing and PCR reactions were made at the Recombinant DNA/Protein Resource Facility, Oklahoma State University. Pneumococcal competence factor was kindly provided by Dr. Donald A. Morrison, University of Illinois, Chicago. Miscellaneous chemicals and reagents were purchased from Fisher, Sigma, USBC, and VWR.

Growth and Storage of Bacterial Strains

Streptococcal strains were grown in casein hydrolysate (CAT) medium (10 g of casein hydrolysate, 5 g tryptone, 5 g NaCl, and 1 g yeast extract in one liter of distilled water). The media was autoclaved, cooled and supplemented with sterile 20% glucose and

TABLE III

CLONING VECTORS AND PLASMIDS

PLASMID	RELEVANT FEATURE	SOURCE
pBluescript (SK$^+$/KS$^+$)	Amp^r, lacZ$^+$	Stratagene
pVA891	Cm^r, Em^r	47
pLS1	Tc^r	76
pET-30 a-c+	Kan^r	Novagen
pMal	Amp^r, lacZ$^+$	NEB
pSP108	3.25 kb *Xba*I :: SK$^+$ *Xba*I	This work
pSP111	1.07 kb *Xba*I :: SK$^+$ *Xba*I	This work
pSP149	0.96 *Eco*RI of pSP111 :: pLS1 *Eco*RI	This work
pSP152	pSP149 with ORF3 mutated at the *Kpn*I site	This work
pSP153	ORF3 :: pLS1 *Eco*RI/*Hin*dIII	This work
pSP154	ORF7 :: pLS1 *Eco*RI/*Hin*dIII	This work
pSP155	pMalP2 *Xmn*I :: 1.1 kb PCR fragment	This work
pSP156	ORF8 :: pLS1 *Eco*RI/*Hin*dIII	This work
pSP157	pET-30 b *Eco*RV/*Hin*dIII :: 1.0 kb *Ecl*136II/*Hin*dIII of pSP155	This work

0.5 M K_2HPO_4 to yield a final concentration of 11 mM and 16 mM, respectively. The supplemented medium was called CATPG broth or agar (1.5% w/v). *S. pneumoniae* cultures were grown to an OD_{550nm} = 0.2 (2.0 x 10^8 cfu/ml). Due to the induction of autolysis at the stationary phase, pneumococcal cultures to be stored were never allowed to grow beyond OD_{550nm} = 0.2. *S. pyogenes* was grown overnight prior to storage. Both the cultures were stored at -80°C with 10% (v/v) sterile glycerol.

E. coli strains were grown in Luria-Bertani (LB) medium (10 g tryptone, 5 g yeast extract, and 10 g NaCl in one liter of distilled water). The pH of the medium was adjusted to 7.5 with NaOH and the medium sterilized. The agar concentration used was 2.0% w/v. For short term storage, overnight cultures of *E.coli* were stored at -20°C with 50% v/v glycerol while long term storage was at -80°C with 10% v/v glycerol.

All bacterial cultures were grown at 37°C unless and otherwise indicated. Each of the above mentioned media was supplemented with the appropriate antibiotic, whenever there was a need to do so. The concentration of the antibiotics used to select bacterial strains used in this study are tabulated in Table IV.

Transformation

E. coli Transformation

Competent *E. coli* cells were prepared and transformed according to Hanahan (31). The competent cells were stored for 2 months at -80°C. Appropriate volumes of transformed cells were plated immediately on LB agar plates containing the selective agent. In case of ampicillin selection, the cells were grown overnight at 37°C with ampicillin, before plating, to reduce the appearance of satellite colonies. For chromogenic

TABLE IV

SELECTIVE ANTIBIOTIC CONCENTRATION

GENOTYPE OR PHENOTYPE	ANTIBIOTIC	CONCENTRATION (μg/ml) STAB PLATE/BROTH	OVERLAY
Streptococcus pneumoniae			
cat (Tn*5252*)	chloramphenicol	5	15
Em^r	erythromycin	3	5
nov	novobiocin	10	10
str	streptomycin	200	200
tet	tetracycline	2	3
Streptococcus pyogenes			
cat (Tn*5252*)	chloramphenicol	5	
Em^r	erythromycin	1	
nov	novobiocin	20	
str	streptomycin	600	
opt	optochin	20	
Escherichia coli			
Cm^r	chloramphenicol	10	
Em^r	erythromycin	200	
Km^r	kanamycin	70	
Amp^r	ampicillin	50	
Tc^r	tetracycline	10	
Spc^r	spectinomycin	200	

differentiation of recombinants, 50 µl of 20 mg/ml 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal), and 20 µl of 20 mg/ml of isopropylthio-β-D-galactoside (IPTG) were used on each LB plates (100 mm).

S. pneumoniae Transformation

S. pneumoniae cells were made competent according to Guild and Shoemaker (30). The competent cells were stored at -80°C in 10% glycerol. For transformation, the cells were thawed on ice-bath and mixed with the transforming DNA (1 ng). Transformation was carried out at 37°C for 30 minutes. Transformation was stopped by adding DNase to a final concentration of 10 µg/ml for 5 minutes at 37°C. Appropriate dilutions of the transformed cells were made in 5 ml CATPG broth with 2% bovine blood and mixed with 5 ml of CATPG agar cooled to 49°C, and poured as an overlay on a 20 ml base CATPG agar. After the cell layer solidified, 10 ml of CATPG agar was overlaid and incubated for 90 minutes at 37°C to allow phenotypic expression (82). The plates were then overlaid with 10 ml CATPG agar containing the appropriate antibiotic and incubated overnight at the same temperature.

Transformation of Pneumococci using Competence factor (CF)

The lyophilized preparation of competence factor was dissolved and diluted in 50 mM potassium acetate buffer, pH 4.6, to a final concentration of 1 µg/ml. To transform pneumococci using the competence factor, 500 µl of stored cells at an OD_{550nm} of 0.2 were

mixed with 5 ng of competence factor and transforming DNA (1 μg). The cells were incubated at 37°C for 45 minutes. Transformation was stopped by adding DNase I to a final concentration of 10 μg/ml for 5 minutes at 37°C. Plating was done as described for natural transformation. When freshly grown cells at an OD_{550nm}=0.05 were used, the cells were pre-treated with 1 mM HCl for 30 minutes at 37°C prior to the addition of CF to inactivate the natural competence factor.

Conjugation

Conjugation between pneumococcal donors and recipients was performed with minor modifications to the method described by Smith and Guild (72). The cells were grown to an OD_{550nm} = 0.2 in Difco CATPG broth supplemented with 0.001% choline chloride. The donors and recipients were mixed at a ratio of 1:5 in the presence of 10 mM $MgSO_4$, 2 mg/ml BSA, and 100 μg/ml of DNase I. Approximately 6 x 10^8 cells were filtered through nitrocellulose filters (Millipore: 13 mm diameter and 45 μm pore size). The cells were placed cell side down on Difco CATPG agar containing 10 mM $MgSO_4$, 2 mg/ml BSA, 1 mM $CaCl_2$, and 70 μg/ml DNase I, and overlaid with the same agar. Each mating was done in triplicates and repeated at least one more time to check for reproducibility. Incubations were carried out at 37°C for 4 hours maximum. The cells were harvested by cutting out both layers of agar with the filter and resuspending each filter in 2 ml of resuspension broth (CATPG containing 10 mM $MgSO_4$, 2 mg/ml BSA, 10 μg/ml DNase I and 10% Glycerol). After vortexing thoroughly, the liquid portion was transferred to a test tube and stored at -80°C for plating later. Appropriate dilutions of the

conjugation mix were plated by the overlay method with selection and scored for the transconjugants as well as the parental strains.

Conjugations between pneumococcal strains and *S. pyogenes* strains were essentially performed the same way except that incubation period ranged between 4-24 h. To screen for the transconjugants (*S. pyogenes*ΩTn*5252*) and the recipients (*S. pyogenes*), appropriate volumes of the mating mixture were spread on the surface of the plate. The donor *S. pneumoniae* were plated by the overlay method of plating. The transconjugants were individually checked for unselected markers by replica plating on selective CATPG agar with 2% bovine blood.

Chromosomal and Plasmid DNA isolation

Chromosomal DNA isolation from Pneumococci

S. pneumoniae cells were grown in CATPG to an OD_{550nm} of 0.4. Ethylenediamine teteraacetic acid (EDTA), was added to a final concentration of 10 mM. After 10 minute incubation at 0°C, the cells were pelleted at 5,000 rpm in a Sorvall GSA rotor at 4°C for 10 minutes. The pellet was washed once in 1X SSC (150 mM sodium chloride and 15 mM sodium citrate) and the cells were resuspended in 4 ml lysis solution {30 mM EDTA, 0.1% Sodium Dodecyl Sulfate (SDS), and 0.1% Sodium Deoxycholate (DOC)}. The suspension was incubated at 37°C until lysis and transferred to 65°C for 15 minutes. Purification of chromosomal DNA from the lysate was by the method described by Marmur (48).

Plasmid DNA isolation from Pneumococci

For small scale plasmid preparations, colonies of putative *S. pneumoniae* clones were grown in 5 ml CATPG broth without selection. The cells were grown to an OD_{550nm} of 0.4 and 3 ml of the culture was used for plasmid isolation. The cells were resuspended in 50 µl 1X SSC. Fifty µl SDS/DOC (0.2% (w/v) SDS and 0.4% (w/v) DOC) were added to the cell suspension and incubated at 37°C until lysis. The lysate was boiled for 5 minutes and 8 µl of the sample was electrophoresed on an agarose gel.

Large scale plasmid purification was done starting from a 250 ml culture of OD_{550nm} = 0.2-0.4. 10 ml of 0.5 M EDTA was added to the culture and the cells were pelleted at 7,000 x g in a Sorvall GSA rotor for 10 minutes at 4°C. The cells were washed once in 30 ml 1X SSC and pelleted before resuspending in 1 ml 0.1X SSC. Four ml of lysing solution containing 1 volume 10X lysis buffer [1% (w/v) DOC, 0.1% (w/v) SDS], 1 volume of 1M Tris-HCl (pH 8.0) and 9 volumes 10X SSC was added and the cells were incubated at 37°C until lysis. Five ml of 4.4% (w/v) SDS solution was added to the lysate [4 volumes of 20% (w/v) SDS, 5 volumes of 10X SSC, and 1 volume of Tris-HCl, pH 8.0] and incubation was continued at 65°C until the lysate cleared. To the lysate, 3.3 ml of 4 M NaCl was added, and it was left overnight at 4°C. The following day, the lysate was centrifuged for 40 minutes at 4°C at 11,000 x g. One ml of RNaseA (1 mg/ml) was added to the supernatant and the supernatant was extracted once with chloroform:isoamyl alcohol (24:1). The plasmid DNA in the aqueous phase was precipitated at -20°C for 15 minutes with 95% v/v ethanol, pelleted and resuspended in

4 ml TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM EDTA). Cesium chloride (Final concentration: 1.05 g/ml) and 400 µl of ethidium bromide (10 mg/ml) were added and centrifuged at 227,640 x g in a VTi65.2 rotor at 17°C for 18 h (or at 383,700 x g for 4 h) in an XL-70 ultracentrifuge (Beckman). The plasmid DNA band was harvested using a 22-G needle, extracted with water saturated butanol to remove the ethidium bromide, and dialyzed overnight in TE buffer.

Plasmid DNA isolation from *E. coli*

Small scale plasmid was prepared by inoculating a putative *E. coli* colony in 3 ml of LB with appropriate antibiotic selection. For the plasmid preparation, 1.5 ml of the overnight culture was used. The plasmid DNA was isolated by the rapid alkaline-SDS lysis method described by Sambrook et al. (62) and resuspended in 200 µl TE buffer. Eight µl of the plasmid DNA was electrophoresed on an agarose gel to check for recombinant plasmids. For restriction endonuclease analysis, the plasmid DNA was extracted using phenol-chloroform prior to ethanol precipitation.

Large scale plasmid purification was performed essentially in the same manner as the miniprep except for the use of a 250 ml culture for scale-up and a CsCl density gradient to separate the plasmid from the genomic DNA. In case of a low copy number plasmid, an additional plasmid amplification step was performed by growing the cells for a further 12 h in the presence of spectinomycin (300 µg/ml). Cesium chloride at 1 g/ml and ethidium bromide at 1 mg/ml were added to the DNA resuspended in TE buffer, and centrifuged at 227,640 x g in a VTi65.2 rotor at 17°C for 18 h (or at 383,700 x g for 4 h) in the XL-70 ultracentrifuge (Beckman). The plasmid DNA band was harvested using a 22-G needle,

extracted with water-saturated butanol to remove the ethidium bromide, and dialyzed overnight in TE buffer.

DNA manipulation

Cloning

The pneumococcal DNA fragments were subcloned in plasmid vectors and transformed into *E. coli* host cells. Ligations were carried out in 20 µl volume with less than 5 µg DNA at a ratio of 1 vector to 5 insert at 16°C for 24 h. For insertion mutagenesis, the insert:vector ratio was 1:1 in a 60 µl volume. For insertion mutagenesis, the ligation mixture was extracted with phenol-chloroform, linearized with restriction enzyme and then used as donor DNA in transformation of competent pneumococcal cells.

Agarose Gel Electrophoresis

DNA samples were analyzed at room temperature by separation on horizontal agarose gels using 1/2X TBE (45 mM Tris-borate, and 1 mM EDTA, pH 8.3). Depending on the size of the DNA, the concentration of the agarose varied from 0.8%-1.2%. The DNA samples were mixed with tracking solution containing 5% (v/v) glycerol, 3 mM EDTA (pH 8.0), bromophenol blue (0.04%) and xylene cyanol (0.04%), before loading. Typically, the gels were run at 50 v constant electric current for approximately 90 min. After electrophoresis, the gel was stained in ethidium bromide (1 µg/ml final concentration) for 10 minutes followed by destaining in deionized water for the same period. The separated DNA samples were observed on a 300 nm UV illuminator. Photographs were taken using a Kodak Polaroid camera. Molecular weight standards (*Hin*dIII fragments of

phage Lambda and *Hae*III fragments of phage ϕX174) were run along with the DNA samples for size comparison. Preparative minigels were used to purify specific DNA fragments, and 0.6%-1.0% gels were made with ultrapure agarose (Bio-Rad). After staining and destaining, a gel slice containing the DNA fragment was electroeluted and purified according to Sambrook et al. (62).

Conversion of 5' overhang to blunt-end with Klenow fragment

This procedure was used to fill recessed 3' ends of double stranded DNA following cleavage by a restriction enzyme. In a situation where there is no alternative restriction site which has a 5' overhang that could be used to create a blunt end using Klenow, the 3' overhang can be converted to a blunt end using the 3'→5' exonuclease activity of Klenow DNA polymerase. The reaction mix consisted of 4.5 μg of digested DNA, 1X dNTP mix (0.1 mM each dNTP), 1X Klenow buffer and Klenow DNA polymerase (1-5 units) in a final volume of 50 μl, to fill in the 3' end. The dNTP was omitted for degrading the 3' overhangs. The reaction was incubated at room temperature for 15 minutes. Following phenol:chloroform:isoamyl alcohol extraction, the DNA was precipitated with 1/2 volume 7.5 M ammonium acetate and 2 volumes ethanol for 20 minutes at -20°C and used in ligation reactions.

Conversion of 3' overhang to blunt-end fragments with T4 DNA polymerase

The presence of dNTP does not affect the exonuclease activity of the T4 DNA polymerase on single stranded DNA. Therefore the following protocol was used for the conversion of 3' overhangs to blunt ends. In a reaction tube, 1 pmol ends 3' extended

DNA fragment, 2 µl 10X T4 DNA polymerase buffer, 2 µl 10X dNTP mix (0.5 mM each dNTP), 1 U T4 DNA polymerase, and distilled water to make up the total volume to 20 µl were mixed and the reaction was allowed to proceed at 37°C for 30 minutes. The DNA was precipitated with 1/2 volume 7.5 M ammonium acetate and 2 volumes 95% ethanol at -20°C for 30 minutes. The DNA was pelleted and resuspended in 20 µl TE. Ligations were performed using this DNA.

SOUTHERN HYBRIDIZATION

Blotting

DNA-DNA hybridizations were essentially performed according to the method described by Southern (73). DNA samples for blotting were first digested using the appropriate restriction enzymes. The fragments were separated on 0.8% agarose gels, as described earlier. The DNA fragments were denatured with 0.5 M NaOH for 30 minutes at RT. The DNA was transferred onto a Genescreen Plus nylon membrane (NEN) using a Model 785 Vacuum Blotter (Bio-Rad). After transfer the membrane was soaked in 2X SSC for 5 minutes, air-dried, and stored in a dessicator under vacuum.

Pre-hybridization

Before hybridization with a radiolabeled probe the membrane with the DNA was placed in a "seal-a-meal" bag to which 10 ml of the prehybridization solution (1g Dextran Sulfate, 0.58 g NaCl, 1 ml 10% SDS and 8 ml ddH_2O) was added. The solution was

spread evenly on the membrane, and after squeezing out air bubbles, the bag was sealed and prehybridization was carried out in a 65°C water bath overnight with gentle shaking.

Nick translation

Labeling of DNA to be used as probe for Southern hybridization was carried out as follows. Probe DNA (0.5-1.0 μg) was nick translated in the presence of 0.5 μl of DNase I (0.5μg/ml), 5 μl of 10X buffered dNTP mix (5 μM each of dATP, dTTP and dGTP), 5 μl {α-^{32}P}dCTP (10mCi/ml), and 0.5 μl of *E. coli* DNA polymerase I (9000U/ml) in a 50μl total volume. The DNA was labeled at 16°C for 2 h. The reaction was terminated by adding 25 μl of 0.5 M EDTA, 25 μl of sheared salmon sperm DNA (2 mg/ml), 50 μl of 7.5 M ammonium acetate and 50 μl of TE. The DNA was precipitated with 100% ethanol for 15 minutes at -20°C, pelleted, dried, and resuspended in 600 μl of TE. The efficiency of incorporation of the isotope was measured by diluting 0.5 μl of the labeled probe in 5 ml of liquid scintillation cocktail (Beckman) and measuring the radioactivity in an LS 6000 SC liquid scintillation counter (Beckman).

Hybridization

Generally, 1-4 x 10^6 CPM (counts per minute) of labeled DNA was mixed with 100 μl 20X SSC, 400 μl sheared salmon sperm DNA (2 mg/ml) and the volume was adjusted to 2.0 ml with ddH_2O in a screw capped tube. The probe was denatured by boiling for 10 minutes. The denatured sample was cooled rapidly by swirling in an icebath

for 3 minutes. The plastic bag containing the prehybridized membrane was cut open and the denatured probe was added into the bag using a pasteur pipet and resealed. Hybridization was carried out in a 65°C waterbath with gentle shaking for at least 18 h. Following hybridization the membrane was removed from the bag and washed twice with 2X SSPE buffer {17.53%(w/v) NaCl, 2.76% (w/v)of NaH_2PO_4, and 0.74% (w/v) of EDTA, pH 7.4} for 15 minutes at room temperature, twice with 2X SSPE, 2% SDS for 45 minutes at 65°C, twice in 0.1X SSPE buffer for 30 minutes at room temperature, and once in 3 mM Tris base (unbuffered) solution for 15 minutes at room temperature. The membrane was briefly air-dried, and exposed to X-ray film (Kodak X-OMAT AR) at -80°C for varying time periods. The exposed film was developed according to the manufacturer's recommendation.

Filter Colony Hybridization

Filter colony hybridizations were performed to identify recombinant clones in the absence of blue/white screening. The colonies were grown on LB agar with selection and picked with a tooth-pick and replica plated on two LB plates of which one served as the master plate. Each plate also had positive and negative controls. After overnight incubation at 37°C, the colonies were transferred to NEN membranes. Each membrane was sterilized by UV light for 5 minutes before placing on the plate with the colonies. This was incubated for 2 h at 37°C to permit the colonies to grow on the filter paper. To lyse the colonies, the filter was lifted off the plate, and placed colony side up in a petri plate containing 0.75 ml of 0.5 M NaOH for 20 minutes. The process was repeated until lysis when the colonies glistened. The filter was neutralized twice with 1 ml neutralization buffer (0.2 M Tris-HCl, pH 7.0 and 0.6 M NaCl) for 10 minutes. Each filter was rinsed

twice with 2X SSC and twice with ethanol. The filters were then air-dried on paper towels and stored in a dessicator under vacuum. Prehybridization and hybridization were the same as described for blots.

DNA Sequencing with T7 DNA Polymerase (Sequenase version 2.0)

Dideoxy chain termination method of sequencing was carried out with Sequenase version 2.0 (USBC) T7 DNA polymerase, and {α-^{35}S}dATP (12.5 mCi/ml) from NEN.

Preparation of Plasmid DNA templates

Double stranded DNA templates were prepared with Wizard miniprep DNA purification systems (Promega) according to the manufacturer's recommendations with some modifications. Ten ml of an overnight culture was pelleted and resuspended in 200 µl of cell resuspension solution (50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 100 µg/ml RNase A). The cells were lysed by the addition of 200 µl cell lysis solution (1% SDS and 0.2 M NaOH) at room temperature for 5 minutes. The lysate was next neutralized with 200 µl of 2.55 M potassium acetate for 5 minutes at room temperature. After centrifugation at top speed in a microfuge (13,000 x g), the supernatant was transferred to a fresh eppendorf tube and 1 ml of DNA purification resin was added to it and mixed by inversion. This mixture was passed through a purification column using a 3 ml disposable syringe. The column was washed once with 2 ml of column wash solution (0.2 M NaCl, 20 mM Tris-HCl, pH 7.5, 5 mM EDTA, and 50% ethanol). The excess wash solution was removed by brief centrifugation and the DNA was eluted with 100 µl TE buffer and stored at -20°C.

For sequencing DNA at the OSU Core Facility, the above described method was followed except with one variation. The column wash solution contained 55% ethanol, 80 mM potassium acetate, 8.4 mM Tris-HCl, pH 7.5, and 40 μM EDTA. This alternative protocol provided DNA that could be used for fluorescent sequencing.

Single-stranded DNA templates were prepared by the method described by Sambrook et al. (62). DNA fragments were cloned in pBluescript vectors (Stratagene) and maintained in *E. coli* JM109 strain. These cultures were grown overnight in 5 ml of 2X YT-broth (10 g tryptone, 10 g yeast extract, and 5 g NaCl per liter of distilled water) with $ampicillin_{50}$ at 37°C for 12 h. This was used as starter culture to inoculate 50 ml of the same broth containing 0.001% thiamine and incubated for 30 minutes at 37°C. Approximately, 10^{11} plaque forming units (PFU/ml) of M13K07, the helper phage was added and incubated for 1 h at 37°C. Fifty ml of fresh media containing 70 μg/ml kanamycin and 50 μg/ml ampicillin were added and incubation carried on overnight. The culture was centrifuged to pellet the bacterial cells and the supernatant containing the f1 phage particles was mixed with 5% (w/v) polyethylene glycol (PEG) and 0.8 M ammonium acetate and left at 0°C overnight. The phage particles were then pelleted by centrifugation at 9,200 x g for 45 minutes and resuspended in 0.5 ml TE buffer. Two phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol extractions were performed before ethanol precipitation and resuspension in 100 μl TE.

Construction of nested deletions for sequencing

Nested deletions were created of DNA fragments over 1.0 kb in size cloned in pBluescript vector. The recombinant plasmid was double digested with two restriction enzymes in such a way that they left a 3'-protruding end near the primer binding site and a

5'- protruding end near the end of the insert DNA. This was because the enzyme exonuclease III used for creating nested deletions has a specific 3'→5' exonuclease activity. The reaction mixture, a total volume of 100 μl, contained approximately 5 μg of double digested DNA, 10 μl of 10X exonuclease buffer (0.6 M Tris-HCl, pH 8.0 and 6 mM $MgCl_2$) and exonuclease III enzyme (450 U/reaction) on ice. The reaction was started by transferring the tube to 37°C water bath and 12.5 μl samples were removed every 2 minutes for 16 minutes and transferred to 0°C. The first four samples were combined in one tube and the last four samples in another before adding 150 μl of S1 nuclease solution (150 U S1 nuclease, 1% glycerol, 60 mM NaCl, 1.3 mM $ZnSO_4$, and 8 mM potassium acetate, pH 4.6). The tubes were transferred to room temperature for 20 minutes. This was done to generate blunt ends because S1 nuclease acts on single stranded DNA. The reaction was terminated with 20 μl of S1 stop buffer (50 mM EDTA and 0.7 M Tris base, pH 8.0). The samples were extracted with phenol-chloroform-isoamyl alcohol and chloroform-isoamyl alcohol followed by ethanol precipitation. The DNA was ligated and transformed into *E. coli* JM109 cells. A number of deletion derivative plasmids were isolated, confirmed by restriction analysis and used for sequencing.

Denaturing the plasmid DNA

The dsDNA (1 μg) was denatured with 0.2 N NaOH and 0.2 mM EDTA at room temperature. It was neutralized with 0.3 M Tris-HCl, pH 4.5, and 0.8 M sodium acetate on ice. The denatured DNA was precipitated with ethanol, pelleted, vacuum dried and stored at -20°C.

Priming of the Denatured DNA

Annealing of template to primer was carried out by setting up a 10 µl reaction volume containing 1 µg DNA, 30 ng primer and 1X Sequenase reaction buffer and incubating it at 37°C for 20 minutes. A number of synthetic primers were constructed by the Recombinant DNA/Protein Resource Facility, Oklahoma State University. T7 promoter primer (Core Facility), M13 forward and reverse primers (Promega), and malE primer (NEB) were also used. ssDNA templates were dealt with in the same manner except that incubation was carried out at room temperature for 30 minutes.

Labeling of the Plasmid DNA

The primed DNA was transferred to 0°C for labeling and 3.5 µl of labeling mix and 2.0 µl of Sequenase version 2.0 were added. Labeling was allowed to proceed for 10 minutes at 0°C. The labeling mix was prepared by mixing 10 µl of $\{\alpha^{35}\text{-S}\}$dATP, 16 µl of 0.1 M DTT and 34 µl of dGTP mix for obtaining long stretches of DNA sequences. For obtaining sequences closer to the primer binding site, 10 µl of dGTP mix, 10 µl of $\{\alpha^{35}\text{-S}\}$ dATP, 28 µl of 0.1 M DTT and 52.0 µl of double distilled water (ddH_2O) were mixed. The enzyme was diluted 1:7 with 1 µl of enzyme and 7 µl of enzyme dilution buffer.

Termination

Four tubes labeled G, A, T, C were placed in a 49°C warming block and to each tube 2.5 μl of the corresponding termination mix (ddNTP) was added. The labeled DNA (3.5 μl) was added to each of the four tubes, microfuged, and incubated for 4 minutes at 49°C. All the tubes were then transferred to room temperature and 4 μl of stop buffer (95% formamide, 20 mM EDTA, 0.05% bromophenol blue and 0.05% xylene cyanol FF) was added to each tube and stored at -20°C until sequencing.

Denaturing Polyacrylamide Sequencing Gel

7% (w/v) polyacrylamide gel containing a concentration gradient in TBE buffer was prepared according to the methods described by Biggin et al. (5). For electrophoresis, a Sequi-Gen Sequencing Cell from Bio-Rad was used. Both glass plates were thoroughly cleaned and air dried. The gel plate was coated with "Glue" (10 ml 95% ethanol, 30 μl Glacial acetic acid, 50 μl of G-methacryloxy propyl trimethoxysilane and 0.5 ml of ddH_2O), and the buffer chamber plate was coated with Sigmacote (Sigma). Gel solution A contained 17.5% (v/v) of 40% acrylamide solution (38% acrylamide and 2% bis-acrylamide), 25% (w/v) urea, and 10% (v/v) 10X TBE buffer. Immediately before pouring the bottom plug as well as the gel, 0.3% (w/v) ammonium persulfate (APS) and 0.05% TEMED (N, N, N',N'-tetramethylethylenediamine) were added to the solution. The plates were assembled and the bottom was plugged with solution B [Solution A supplemented with 25% (w/v) of sucrose and 0.005% (w/v) of bromophenol blue]. The gel apparatus was then filled with the denser solution B at the bottom of the gel at a ratio of

1:5 (solution B: solution A) and the chamber was filled with solution A. The gel was allowed to solidify at room temperature for at least 18 h.

Denaturing Polyacrylamide Sequencing Gel Electrophoresis

Following polymerization, the gel was prerun for 1 h or until the temperature reached 40-45°C, using 0.5X TBE in the chamber and 1X TBE in the bottom tray at 35 Watts constant power. Before loading, samples were denatured in a 95°C water bath for 2 minutes and immediately loaded on the gel. To get effective resolution of the sequences, 3 M sodium acetate was added to the bottom tray to yield a final concentration of 1 M after the bromophenol blue in the sample ran off. At the completion of electrophoresis the gel apparatus was dismantled and the glass plate with the gel was fixed for an hour at room temperature in 10% glacial acetic acid. The gel was briefly rinsed with distilled water and dried overnight at 65°C. The gel was exposed to X-ray film (Kodak XAR-5 or BIOMAX MR) until sufficient exposure was obtained. The film was removed, developed and fixed according to the manufacturer's recommendations. The sequences were manually read by placing the autoradiogram on an illuminator.

Nucleotide Sequence Analysis

To analyze DNA sequences for homology alignment, restriction sites, identification of open reading frames and prediction of amino acid sequences, the UWGCG Sequence Analysis Software Package (Genetics Computer Group, Inc., Wisconsin) was used. Homology searches were performed by using the MacVector 3.5 software program and searching the DNA sequences in various data banks. The deduced amino acid sequences of

potential open reading frames were also compared with sequences in data bases via the University of Oklahoma Computer Center.

DNA Amplification with the Polymerase Chain Reaction (PCR)

DNA amplifications were performed with Deep Vent DNA polymerase. The basic PCR protocol was as recommended by the manufacturer with some modifications. PCR assays were done in 100 µl total volumes in 0.5 ml microcentrifuge tubes. Transposon DNA from the streptococcal species under study served as the template DNA. A typical reaction mixture contained 10-100 ng of template DNA, 2 primers each at 0.2-1.0 µM, 1X concentration of Vent reaction buffer {10 mM KCl, 2 mM $(NH_4)SO_4$, 20 mM Tris-HCl (pH 8.8), 2 mM $MgSO_4$ and 0.1% Triton X-100}, 200-400 µM each dNTP and 0.75 U Deep Vent DNA polymerase. The reaction mixture was overlaid with 100 µl sterile mineral oil, briefly centrifuged, and PCR performed in a Coy Tempcycler Model 60 (Coy Laboratory Products, Inc.).

On an average, 30 cycles of amplification were carried out, each consisting of a 1 minute denaturation period at 94°C, 45 second annealing at a temperature which was 5°C lower than the calculated melting temperature of the primer with the lower Tm, and a variable extension period at 72°C. The Tm (also called Td; dissociation temperature), was calculated by the relation, Tm = 4(G+C) + 2(A+T), where the letters stand for the number of each nucleotide in the primer. The extension period was determined by allowing 1 minute per kilobase of DNA at 72-75°C and, therefore, depended on the length of the DNA segment to be amplified.

Five μl of the amplified DNA was checked on an agarose gel to assess the quality and quantity of the amplified product.

Purification of PCR products

PCR products were cleaned with Geneclean II (Bio 101) according to the manufacturer's suggestion. After PCR, the reaction mixture was transferred to a new tube leaving behind the mineral oil. To this, 3 volumes of NaI (6 M) and 15 μl of Glassmilk were added, mixed and adsorbed at 0°C for at least 5 minutes. The adsorbed DNA was pelleted at full speed for 10-20 seconds and resuspended in 500 μl wash buffer (50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 2.5 mM EDTA, and 50% ethanol). The DNA was pelleted and the wash step repeated twice more. The final wash buffer was completely removed, and the pellet resuspended in 50 μl sterile TE buffer, and the tube placed at 65°C for 5-10 minutes to elute the DNA. The sample was centrifuged for 2 minutes and the supernatant containing the eluted DNA was transferred to a fresh tube. Samples (2 μl) were always checked on an agarose gel before proceeding with cloning.

Protein Expression

In vitro Transcription and Translation

The two stages of protein expression, transcription and translation, are delineated by systems for synthesizing proteins *in vitro*. Such systems can be prepared in the form of cell-free extracts, by breaking cells and centrifuging the mixture to remove matter such as cell walls, membrane fragments, and nuclei (from eukaryotic cells). When provided with a

suitable source of energy and precursors, the "supernatant" or components purified from it can translate most mRNAs that are added to it into protein products. The *in vitro* transcription and translation assays were performed to identify protein products that could be made from the DNA fragment under study and to verify which of the ORFs present in the sequenced region actually code for a functional protein.

Single Tube Protein™ System 2

The single tube protein system 2 (STP2) from Novagen is designed for efficient in vitro synthesis of proteins directly from supercoiled or linear DNA templates containing a bacteriophage T7 RNA polymerase promoter. The system is based on transcription with T7 RNA polymerase followed by translation in an optimized rabbit reticulocyte lysate. A standard reaction consists of a DNA template (0.5 μg plasmid or 2 μl PCR amplification reaction) which is transcribed at 30°C for 15 minutes followed by the addition of translation mix and continued incubation for up to 90 minutes. Procedures followed were according to the manufacturer's recommendation.

Transcription Reaction

The standard transcription reaction volume was 10 μl. Eight μl of STP2 T7 Transcription mix, 0.5 μg plasmid DNA free of RNase, Mg^{2+} and salts, and nuclease-free water to make up the volume to 10 μl were mixed in a microfuge tube, stirred with a pipet tip, and the transcription reaction carried out at 30°C for 15 minutes.

Translation Reaction

The translation reaction consisted of 40 μCi ^{35}S-methionine, 30 μl of STP2 translation mix and nuclease-free water to make up the volume to 40 μl. This was added to the transcription mix, gently stirred with a pipet tip, and the reaction was allowed to proceed for 60 minutes at 30°C.

Control Reactions

A blank reaction without added DNA served as a negative control to measure the amount of background incorporation of labeled amino acid. A reaction with STP control DNA was also included to verify the performance of the experiment.

Analysis of Translation Products

After the translation incubation step, 1 μl of a 10 mg/ml RNase A was added to the reaction mixture and incubated at room temperature for 5 minutes. This was done to stop the reaction and to effectively remove charged tRNAs which can appear on protein gels. Acetone precipitation was performed to remove unincorporated label which may give a background. Essentially, to the above reaction tube, 100 μl of 1% SDS was added followed by the addition of 0.9 ml of acetone. After vortexing to mix the contents, the tube was left at room temperature for 2 minutes, immediately followed by microfuging for 2 minutes (13,000 x g). The pellet was washed in 0.5 ml of ice-cold acetone and spun again for 4 minutes. The pellet was dried at 65°C for 15 minutes, and dissolved in 100 μl

of dissolving solvent (0.1 M Tris, pH 8.0, 2% SDS and 1% 2-mercaptoethanol). The resuspended proteins were mixed with 100 µl of SDS-PAGE sample buffer (60 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 0.02% bromophenol blue, and 1% 2-mercaptoethanol). The samples were heated at 95°C for 5 minutes and appropriate volumes were loaded on an SDS polyacrylamide gel.

E. coli S30 Extract system for circular DNA

The *E. coli* S30 extract system provides a powerful tool for identifying and characterizing polypeptides by allowing transcription/translation of DNA sequences cloned in plasmid or lambda vectors. The S30 system contains an S30 premix lacking cystiene, methionine or leucine and contains all other required components, including NTPs, tRNAs, an ATP regenerating system, IPTG and appropriate salts. This allows for the radiolabeling of translation products. For this study, we used the DNA cloned into pBluescript vectors where the cloned genes were under control of T7 RNA promoter.

Coupled Transcription-Translation Procedure

The standard reaction consisted of 4 µg of DNA template, 5 µl amino acid mixture minus methionine, 20 µl S30 Premix without amino acids, 1 µl {^{35}S}methionine (1,200 Ci/mmole at 15 mCi/ml), 15 µl S30 Extract and nuclease-free water to bring the final volume to 50 µl. After mixing the components by a quick spin, the reaction was allowed to proceed for 2 hours at 37°C. The reaction was stopped by transferring the tubes to 0°C for 5 minutes.

A positive control reaction was included using the pBEST*luc* DNA. A negative control with pBluescript SK+ alone, the vector used for the cloning of the passenger DNA, was also included in the protocol.

Gel Analysis of Translation Products

Once the S30 extract reaction was completed, a 5 µl aliquot was removed for acetone precipitation. Twenty µl of acetone was added to the reaction mix and incubated at 0°C for 15 minutes. The acetone-precipitated S30 sample was centrifuged at 12,000 x g for 5 minutes. The pellet was dried for 15 minutes under vacuum, resuspended in 20 µl sample buffer, and heated at 100°C for 5 minutes before loading on an SDS polyacrylamide gel.

In vivo Transcription and Translation

^{35}S labeling of proteins after infection and induction by phage and IPTG

Radioactive labeling of plasmid-specific products was carried out essentially as described by Tabor et al. (79) with the following modifications. Cells carrying pBS-SK+ derivative recombinant plasmids were grown at 37°C in 2X YT medium [1% (w/v) yeast extract, 1.6% (w/v) tryptone] supplemented with 0.001% (w/v) thiamine. At OD_{550nm} = 0.4, the cells were infected with M13 mGP1-2 at a multiplicity of infection (m.o.i) of 40. After shaking for 30 minutes at 37°C, a 5 ml aliquot of infected cells were centrifuged, the pellet washed twice, and resuspended in 10 ml M10 medium supplemented

with 0.1% glucose (w/v), 20 μg/ml thiamine, and 200 μg/ml each of 17 amino acids, omitting methionine, cysteine and leucine. After shaking at 37°C for 40 minutes, IPTG at a final concentration of 2 mM, was added to the cells to induce expression of the T7 RNA polymerase gene present in M13 mGP1-2. After growth at 37°C for 30 minutes, rifampicin was added to a final concentration of 300 μg/ml and the cells transferred to 42°C for 40 minutes. One ml aliquot of the culture was radiolabeled with 10 μCi of {^{35}S}methionine (1000 Ci/mmole) and 10 μCi of {^{3}H} leucine (152 Ci/mmole) for 15 minutes at 30°C. The cells were sedimented by centrifugation for 1 minute and the pellet resuspended in 100 μl of SDS-PAGE sample buffer. The cells were lysed by heating at 100°C for 3 minutes and the lysates stored at -20°C until electrophoresis.

Discontinuous SDS-Polyacrylamide Gel Electrophoresis (PAGE)

Discontinuous SDS-PAGE was performed as described by Laemmli (41). A stock solution of 22% (w/v) acrylamide A (BRL) was prepared by dissolving 100 g acrylamide and 2.7 g bis-acrylamide (Fisher) in a total volume of 500 ml of ddH_2O and stored at 4°C. The concentration of acrylamide in the separating gel varied from 12-17% (w/v) depending on the size of the proteins to be separated; however, the stacking gel was always 3% (w/v). To make the electrophoresis mixture for the separating gel, 10.0 ml of 1.5 M Tris-HCl, pH 8.8, 0.4 ml of 10% SDS, a calculated volume of acrylamide A to yield the required concentration and ddH_2O to make a final volume of 40 ml were mixed and deaerated for 15 minutes. Before pouring the mixture into the gel casting assembly, 0.4 ml of 5% ammonium persulfate (APS) and 40 μl of TEMED were added. The acrylamide was

overlaid with water-saturated butanol and allowed to polymerize for 30 minutes. The stacking gel was made up by mixing 1.35 ml Acrylamide A, 0.1 ml 10% SDS, 2.5 ml of Tris-HCl, pH 6.8 and 6 ml of ddH_2O. After deaeration, 150 µl APS and 15 µl TEMED were added and poured over the separating gel. Electrophoresis was carried out in Tris-Glycine-SDS buffer at 20 mA through the stacking gel and 45 mA through the separating gel. Typically one run was completed in 3-4 h. For rapid screening of the protein products, the mini-protean II gel electrophoresis apparatus (Bio-Rad) was used which had a run time of less than an hour.

Samples for electrophoresis were prepared by either pelleting 1 ml of cells and resuspension in 100 µl 1X sample buffer or, if collecting fractions, 100 µl of the fraction was mixed with an equal volume of 2X sample buffer. Samples were boiled at 95°C for 5 minutes and stored at -20°C until electrophoresis. Broad range (2-212 kDa) protein markers (NEB) were also run along with experimental samples as a reference for size determination in each gel. To visualize unlabeled proteins after electrophoresis, gels were stained overnight in 50% ethanol, 10% glacial acetic acid, and 0.1% Coomassie brilliant blue R. The gels were then destained in warm 7% glacial acetic acid.

Fluorography

Following electrophoresis, labeled protein bands were visualized by fluorography. Fluorography dramatically increases the sensitivity of detection of labeled proteins. The increased sensitivity of fluorography is obtained by infusing an organic scintillant into the gel. The scintillant converts the emitted energy of the isotope to visible light and so increases the proportion of energy which may be detected by X-ray film. Fluorography was carried out according to the method of Laskey (43). In brief, the gels were soaked

thrice for 30 minutes each time in DMSO (Dimethyl sulfoxide) followed by soaking in 22% 2, 5-Diphenyloxazole (PPO)/DMSO for 3 h. The gels were finally washed in ddH_2O for 1 h and vacuum dried on a 3 MM Whatman filter paper. The dried gels were exposed to X-ray film (Kodak X-OMAT AR) at -80°C and the film was developed following the recommendations of the supplier.

Large Scale Protein Purification

Protein Purification Using the pMAL system

The appropriate plasmids were used for purification of the fusion protein from one liter culture by affinity chromatography. In brief, 10 ml of an overnight culture of *E. coli* cells carrying the expression plasmid was inoculated in 1 liter of rich broth (10 g tryptone, 5 g yeast extract, 5 g NaCl, 2 g glucose/ liter), supplemented with 100 μg ampicillin per ml. Cells were grown to an OD_{600} of 0.5 at 37°C. One ml of sample was pelleted and resuspended in 50 μl SDS-PAGE sample buffer (uninduced sample). IPTG was added to the remaining culture to give a final concentration of 0.3 mM and incubated for an additional 3 h. At the end of each hour, a 0.5 ml sample was taken, pelleted and resuspended in 100 μl of SDS-PAGE sample buffer. These three samples constituted the induced samples.

At the end of 3 h, the cells were harvested by centrifugation at 5,000 x g for 10 minutes, resuspended in 400 ml 30 mM Tris-HCl pH 8.0, 20% sucrose, and 1 mM EDTA, and were kept shaking at room temperature for 20 minutes. The sample was centrifuged at 9,000 x g at 4°C for 30 minutes and the pellet resuspended in 400 ml 5 mM MgSO4 and

1 mM 2-mercaptoethanol, and placed on ice for 15 minutes. The sample was centrifuged at 9,000 x g at 4°C for 30 minutes and the supernatant saved. This constituted the osmotic shock fluid, and to this 8 ml of 1 M Tris-HCl, pH 7.4 was added. A sample of the osmotic shock fluid was also taken for SDS-PAGE analysis. Ten ml of amylose resin was added to the supernatant and the sample was left at 4°C with constant shaking for 12 h. The preparation was centrifuged and the protein-bound amylose resin was washed twice in 1X column buffer (final concentration 20 mM Tris-HCl, pH 7.4, 200 mM NaCl, 1 mM EDTA and 1 mM DTT) before resuspending the resin in 5 ml of 1X column buffer.

Affinity Chromatography

A 2.5 x 10 cm column was packed with the protein-bound amylose resin and the column washed with 5 column volumes of 1X column buffer. The fusion protein was eluted with 1X column buffer + 10 mM maltose. One and one half ml fractions were collected at a flow rate of 1.5 ml/8 minutes. The protein concentration in each of the fractions was determined using the Bradford assay (6; below). Fractions containing the protein of interest were pooled, concentrated using the Centriprep-10 Concentrator (Amicon) and stored at -80°C to be used for the studies described in Chapter IV.

Purification of Target Protein Using the pET system

The proteins of interest to this study were also purified in large quantities using the pET system. The yield was better and the fusion protein size much smaller than that of the pMAL system. The method used was as suggested by the manufacturer. Briefly, a 400 µl inoculum from a glycerol stock was inoculated into 40 ml of rich broth and incubated with

shaking until OD_{600} = 0.6. The culture was then stored at 4°C overnight. The following day, 20 ml of the overnight culture was spun and the pelleted cells were used to inoculate 1 liter of rich broth. The culture was incubated for 3 h at 37°C to an OD_{600} = 0.6. A 1 ml sample was removed, the cells pelleted and resuspended in 100 µl of 1X sample buffer and this served as the uninduced control. The remaining culture was induced with IPTG at a final concentration of 1 mM and incubated for 3 h. A 1 ml sample was collected, pelleted, and resuspended in 100 µl of 1X sample buffer at this time point to compare the level of expression with that of the uninduced sample. The flask was transferred to ice for 5 minutes and the cells were pelleted by centrifugation at 5000 x g for 5 minutes at 4°C. The cells were resuspended in 60 ml 1X Binding buffer (5 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9) with 0.1% triton and stored at -20°C overnight. After thawing, the sample was sonicated for 3 minutes in 15 second bursts at 12 cycles with the tube in an ice-water bath to avoid heating of the sample which may lead to degradation of the proteins. A 100 µl sample was removed and mixed with 2X sample buffer. Sonication was continued until the sample was no longer viscous. The lysate was centrifuged for 20 minutes at 12,000 x g to remove the debris. Fifty µl of the supernatant was mixed with 50 µl of 2X sample buffer.

Column Chromatography

For the soluble protein preparations, the supernatant was filtered through a 0.45 micron membrane to avoid clogging of the column. The column (His. Bind metal chelation resin) was loaded with the prepared extract and was washed with 10 volumes of 1X

Binding buffer. The flow rate was maintained at 10 column volumes per hour. Fifty µl of the unbound sample was mixed with 50 µl of sample buffer to check for efficiency of binding. The column was then washed with 6 column volumes of 1X Wash buffer (60 mM imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). The bound protein was eluted with 6 column volumes of 1X Elute buffer (1M imidazole, 0.5 M NaCl, 20 mM Tris-HCl pH 7.9). Ten µl of each of the fraction was used to perform a Bradford assay to determine the concentration of the purified protein in the fractions. Fifteen µl of each of the samples collected during the course of the process and the different fractions were loaded on a 17% SDS-PAGE gel to check the efficiency of expression and purification and to check for the presence of the protein.

Processing the purified sample

The fractions containing the protein peak were pooled together and dialyzed to concentrate the protein as follows: The protein containing fractions were transferred to a dialysis bag and dialyzed overnight at 4°C in 2 liter buffer containing 20 mM Tris-HCl (pH 7.4), 1 mM EDTA, 0.1% triton-x-100, 0.05% sarkosyl, 100 mM NaCl, 50 mM KCl, 1 mM 2-mercaptoethanol, 10 µM PMSF and 50% glycerol. The protein was stored at -80°C for further experimentation.

Protein Concentration Determination

Bradford Assay

The Bio-Rad Protein Assay, based on the method of Bradford (6) was the method of choice for determining protein concentration. A standard curve for the assay was plotted

based on known concentration of bovine serum albumin. Protein assay samples were prepared by mixing 100 µl of the protein fraction, 700 µl of ddH_2O, and 200 µl of the dye reagent concentrate. After vortexing thoroughly, the mixture was allowed to incubate at room temperature for at least 5 minutes. The samples were then transferred to disposable polystyrene cuvettes (Bio-Rad) and the absorbance measured using a spectrophotometer (Spectronic 1001, Milton Roy company) set at 595 nm. Comparison to the standard curve provided a relative measurement of protein concentration.

CHAPTER IV

A REGULATORY REGION IN THE CONJUGATIVE TRANSPOSON, Tn*5252*.

RESULTS AND DISCUSSION

We have been studying the biology of Tn*5252* (47kb), a complex composite transposon that is often found carrying a Tn*916*-like element in a region which does not seem to have any transfer functions (4, 38) (Figure 1). While the Tn*916* class of elements integrate at several locations, Tn*5252* prefers to integrate at a unique site in the host chromosome (85). However, extensive homology between the target regions of the different hosts was not detected. Recently, it has been shown that a core sequence of 33 bases in the pneumococcal genome serves as an *att* site for this element (3, 84). A copy of the *att* sequence was also present as part of the transposon and, following integration , the chromosomal *att* sequence formed the right junction and the transposon copy the left (84). The left end of the transposon contains an open reading frame (ORF1) whose protein product has been implicated as the integrase mediating site-specific integration of the element, based on its similarity to other site-specific recombinases as well as available genetic information (38). Subsequently, several transfer related regions were identified by creating insertion/deletion mutations within Tn*5252* and testing the donor potential of the mutants created (38).

I undertook experiments involving DNA sequencing and mutagenesis of the region next to the integrase gene so as to gain a better understanding of the details of its molecular organization and role in the biology of Tn*5252*. A restriction map of Tn*5252* showing the 4.4 kb region of DNA fragment under study is highlighted and presented in Figure 2.

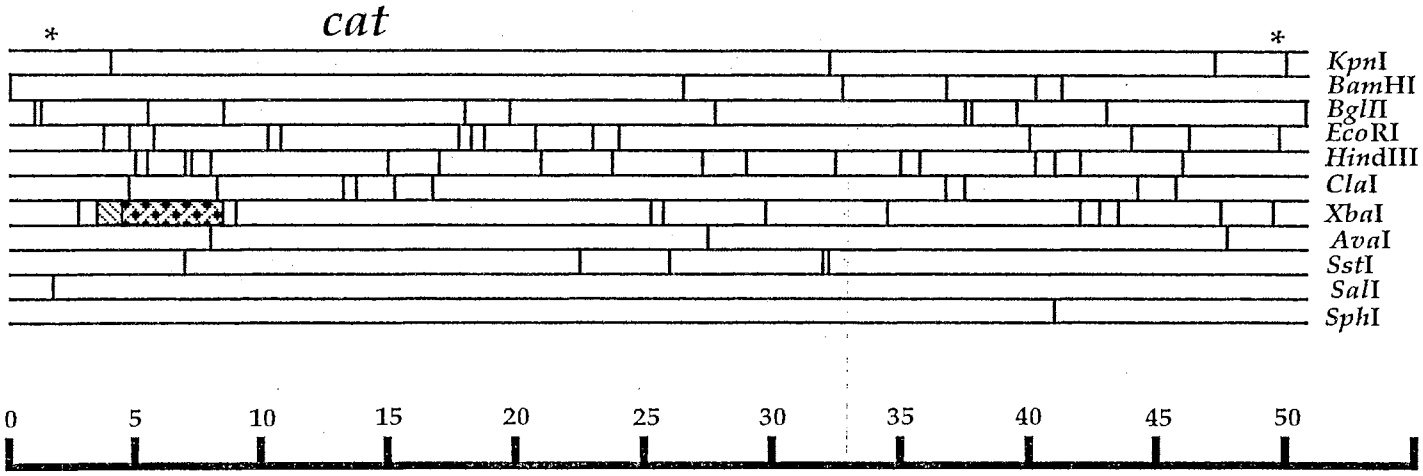


Figure 2. The restriction endonuclease map of Tn*5252*. The region of interest (a 1.07 kb *Xba* I fragment and an adjacent 3.25 kb *Xba*I fragment) is highlighted. The asterisk marks the end of the element.

Construction of Recombinant Plasmids and Sequencing

The *E. coli* plasmid, pVJ15 (85) carrying about 10 kb region from the left junction was used to subclone a 1.07 kb *Xba*I fragment between coordinates 3.5-4.6 of the transposon (Figure 2)) into pBluescript SK+ and KS+ vectors to create the recombinant plasmids, pSP111 and pSP112, respectively. The recombinant plasmid pSP111 was digested with *Kpn*I resulting in two fragments due to the presence of a unique site in the passenger DNA and another in the vector polylinker region. *Kpn*I was used to delete the 0.5 kb *Kpn*I DNA fragment, and the larger fragment was self-ligated to yield pSP118. Similarly, the 0.6 kb *Kpn*I DNA fragment of pSP112 was deleted after *Kpn*I digestion and the larger fragment was self-ligated to yield pSP119. Further plasmid subclones were created after exonuclease III and S1 nuclease digestions of pSP118 and pSP119 followed by self-ligations. The recombinant plasmids, pSP118 and pSP119 and the nested set of deletion derivatives of these plasmids were used for dideoxynucleotide sequencing. A schematic representation of these constructs and the sequencing strategy are given in Figure 3. Double stranded and single stranded templates were prepared from these plasmids and used in sequencing reactions following the protocols described in the materials and methods section. For maximal confidence, the DNA sequence of both strands was obtained.

Sequence Features

The sequence of the 1.0 kb *Xba*I fragment was found to be 1070 base pairs long with a G+C content of 29.8%. Computer analysis of the nucleotide sequence (Figure 4) revealed four open reading frames (ORFs) designated ORF3, ORF7, ORF8, and ORF31 in this region (2, 74). All the ORFs except ORF31 were found to use ATG as the start

Figure 3 Map of the left terminal region of Tn*5252* in *S. pneumoniae* SP1000 and the sequencing strategy. Relevant restriction endonuclease sites are shown. Thin line, chromosomal DNA; J_L, left junction of Tn*5252*; box, tranposon DNA; filled box, DNA that was sequenced. The primary sequence data of the 4.45 kb DNA under study (highlighted in the figure) was obtained using nested set of deletion derivatives of clones containing the 1.07 and 3.25 kb XbaI fragments (pSP111 and pSP108, respectively). The deletion derivatives were obtained following exonuclease III and S1 treatments. With these, sequence-specific primers were designed for further sequencing reactions. For each primer used, the extent of the sequence obtained is indicated by an arrow below. The direction and length of the ORFs described ion the text, are shown above the restriction map.

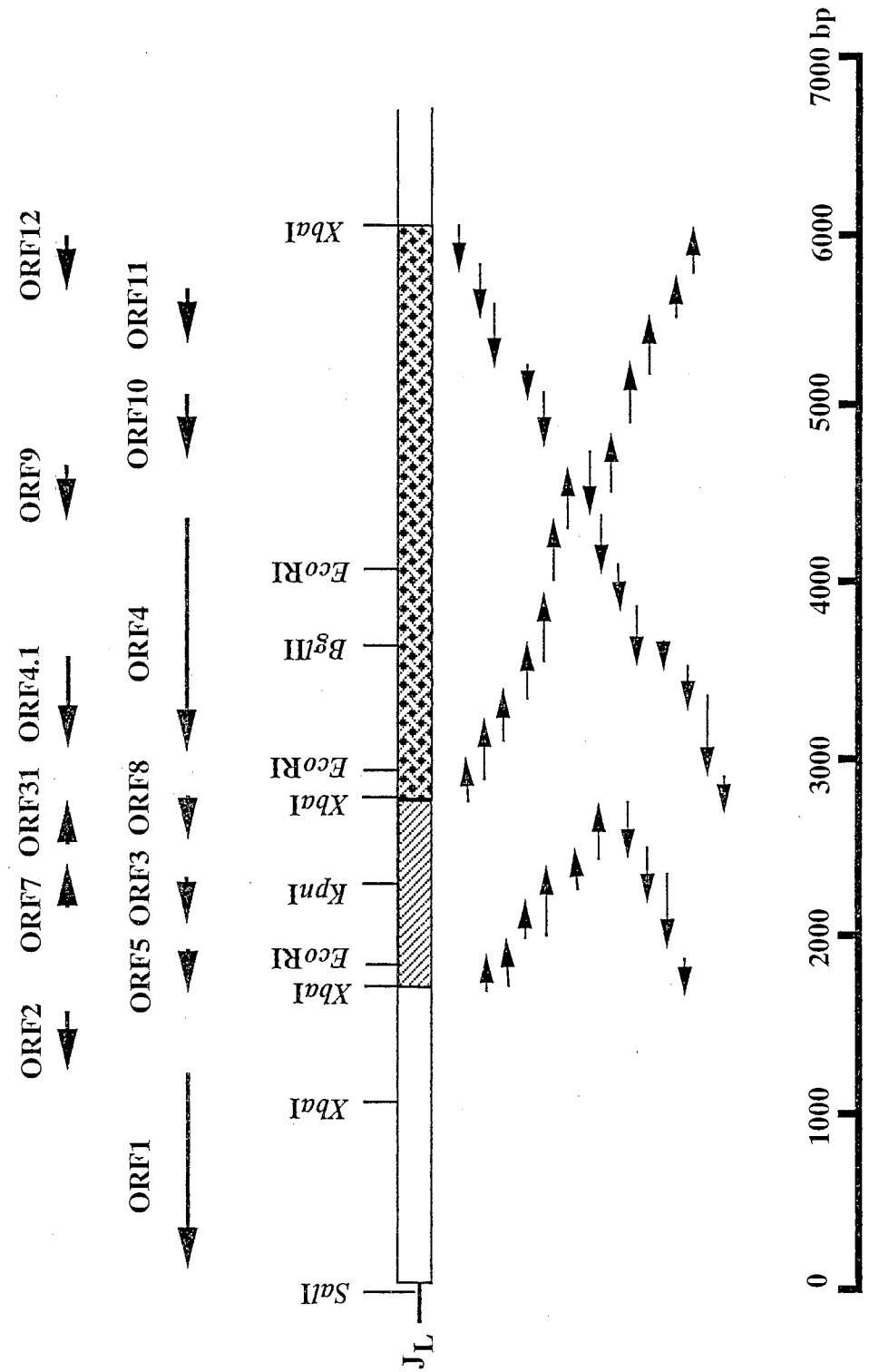

ORF12
ORF9
ORF10
ORF11
ORF4.1
ORF4
ORF31
ORF8
ORF7
ORF3
ORF5
ORF2
ORF1
XbaI
EcoRI
BglII
EcoRI
XbaI
KpnI
EcoRI
XbaI
XbaI
SalI
JL
0
1000
2000
3000
4000
5000
6000
7000 bp

Figure 4. Nucleotide sequence of the 1.07 kb *Xba*I Fragment in Tn*5252* and the amino acid sequence of its products. The 3'-end of the sequence is closest to the left end of the element. The sequence beyond the last *Xba*I site was taken from a previous report (12) for the sake of continuity of ORF5. Relevant restriction sites are given. Potential ribosome-binding sites are underlined. Designated open reading frames are specified at the beginnings of the corresponding amino acid sequence. The -10 and -35 regions of potential promoter sequences are labeled.

*Eco*RI
1 GAATTCCTCTTAGGCAAAATTAAAGAAAAAATAGAACAATTAGATTTTCTTATATCTGCG
61 GAAAATAGTTCGAATGATTTTGAAGATATAACAAATGATTTCATTGCCCAAATATCATAT
*Xba*I -35 **PORF8** -10
121 CTAGAGAATATGATTGAACTAGTCCAAAATAAAATTAATGATTTAACTAATTTAGAGGAA
181 GTATTGTTGAAAGATACGACAAATAGTTCTAGCAATTTAGAAAATAGTATTCAAGGTAAA
ORF8 > L L K D T T N S S S N L E N S I Q G K
241 AGTTCAGTAGATACAATAGAGAAGGATTTATACATATATAAAGGAAAGATTGAAACACTG
S S V D T I E K D L Y I Y K G K I E T L
301 AAGGAACAACATAGAGAAGCAATAAATTTATTTGAAATGTTTAATAAAACAATAAAGAAA
K E Q H R E A I N L F E M F N K T I K K
361 TATAAGGAAAAACAAAATATGAAATCTATTAAGGAAAATGAGATACATTTAGAGTAACAG
Y K E K Q N M K S I K E N E I H L E *
-35
421 TTTCCTAAAAGGTATATATAGAATAGTTCATGTGCCAATTTGTCACATATTTAAAAATAA
PORF3 -10
481 GAAAATAACTATATTATACTTGTTTTAGGAGACATTGGATGTTGAAAAGGATTAGAGATT
ORF3 > M L K R I R D
541 TACGCGAGGATGATGATTTGACACAAGAATATGTTGCAAAAACAATCTTAAATTGTACAA
L R E D D D L T Q E Y V A K T I L N C T
*Kpn*I
601 GATCAGCGTATTCTAAAATGGAATCAGGTACCAGGTTAATCTCTATAGATGACCTTATCA
R S A Y S K M E S G T R L I S I D D L I
661 AACTTGCAGATTTTTATAATGTAAGTTTAGATTACCTTGTAGGTCGAGTGGATAATAAAG
K L A D F Y N V S L D Y L V G R V D N K
721 AAGACCATTACTCCAAAAAGTATTAGGTTAAGAAAGCACATTGACAATTGAATAGTCCAA
E D H Y S K K Y *
* S L V N V I S Y D L

781 AATGGTACTTTCCTCATTTGTGGAGCAGTTTTGAATGGCTCGCCATGATAAGAGCGATAT
I T S E E N T S C N Q I A R W S L L S I
841 TAAAACCATCAATAAAATAGAGCGATACTTTATATGCCATGATACAATGATATACATGAT
L V M L L I S R Y K I H W S V I I Y M < **ORF7**
-35
901 ACTTCTGACCGTTCAGGCTGCACGTGAAGAGCACGAGTGAATCTATGAGTACTTCATCAG
PORF5 -10
961 TCATGCATGGAGGAGAAAATATTTTTTAGGAAGGATGATCGAATGAATATAAAATATATA
ORF5 > M N I K Y I
*Eco*RI
1021 TCTGTCCGAAAATTCCATTGCAGAATGGCGTATAAAAAAGGAATTCTAAAAAGATGGGAT
S V R K F H C R M A Y K K G I L K R W D
1081 GGTTTAAATAAATATACATTGAACAGATGGATTAAAGAAATGCGAGAAAACAGAACATTT
G L N K Y T L N R W I K E M R E N R T F
*Xba*I
1141 TCCATGTATGTAATTAACCCAACCCATAAACTTGTTTTCATTAATCTAGAAGGATTTGAA
S M Y V I N P T H K L V F I N L E G F E
1201 AGTTTCTTAAGATGGAAGCAAAAGCGGACAAAATAAGGAAGTCATGGTATAATATAGGGT
S F L R W K Q K R T K *
1261 AGTTATTTATTCTATACAACAATGATTTATTTCTTTTTAAGAGTTAAGAGGAATTAAATT
1321 ATGTATTATGTAACTAAAACAAATTCAAAAGGGCAACCCTTATATCAAGTGGTTGA

codon. TTG served as the start codon for ORF31. TAG appeared to serve as the stop codon for ORF3, TGA for ORF31 and TAA for ORF7 and ORF8. The predicted products of ORF3, ORF7, ORF8 and ORF31 were approximately 8.9 kDa, 5.7 kDa, 9 kDa and 8.7 kDa, and the pI's 5.7, 7, 8.2 and 6.78, respectively. A fifth open reading frame, ORF5, was also present in this region coding for a protein with a predicted molecular weight of 9.6 kDa and pI 10.7. The carboxy terminal end of the ORF5 extends into an adjacent fragment towards the left terminus of the transposon. Consensus sequences that match the ribosomal binding sequences (RBS) found in gram-positive bacteria (28) preceded each of the ORFs at appropriate locations. While three ORFs were found to be transcribed in one orientation, ORF7 and ORF31 seemed to be transcribed in the opposite orientation. Each of the ORFs was also preceded by a potential promoter sequence. The potential -35 and -10 regions for four of the five ORFs are shown in Figure 5 (74).

Homology Search

The deduced amino acid sequences of all the five ORFs were analyzed for similarities with other protein sequences in the GenBank database. The predicted protein products of ORF7, ORF8 and OR31 did not exhibit sufficient similarity to any sequences in the database. However, the protein product of ORF3 was significantly similar to the *xre*-1 repressor protein of the defective prophage, PBSX, found in *Bacillus subtilis* (34% identity, 51% similarity) (87) and the *ans*R repressor of *B. subtilis* (34% identity, 55% similarity) (78) and weakly homologous to the *cI* repressor protein of the lactococcal temperate phage, Tuc2009 (81) and the predicted protein of *pvu*II*C*, the regulatory gene of *Pvu*II endonuclease (80) (Figure 6). The predicted protein of ORF3 showed the presence of a helix-turn-helix motif suggesting a DNA binding property. The possible protein product of ORF5 showed strong homology, curiously, to the excisionase of the conjugative transposon, Tn*5276* from *Lactococcus lactis* NIZO R5 (44% identity, 65%

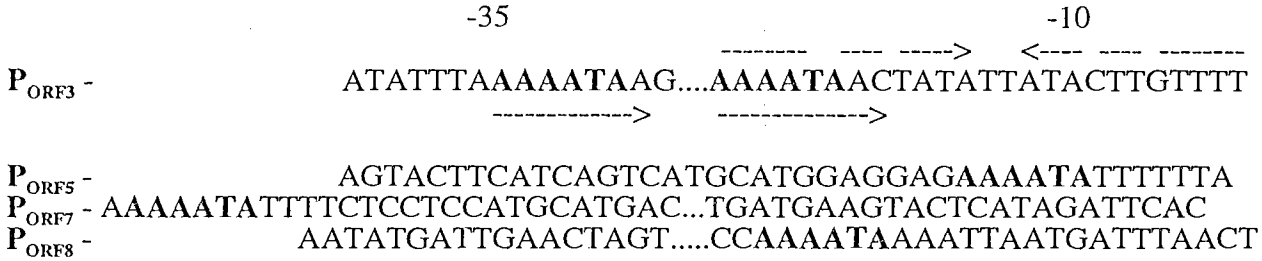


Figure. 5. Alignment of the potential promoter regions, P_{ORF3}, P_{ORF5}, P_{ORF7}, and P_{ORF8}, located within the sequence presented in Figure 4. Regions of dyad symmetry are indicated by arrowed lines above the sequence, while direct repeats are shown by arrowed lines below the sequence. Positions of -35 and -10 promoter consensus sequences are shown at the top. The identical sequence AAAATA present in all these regions is shown in bold.

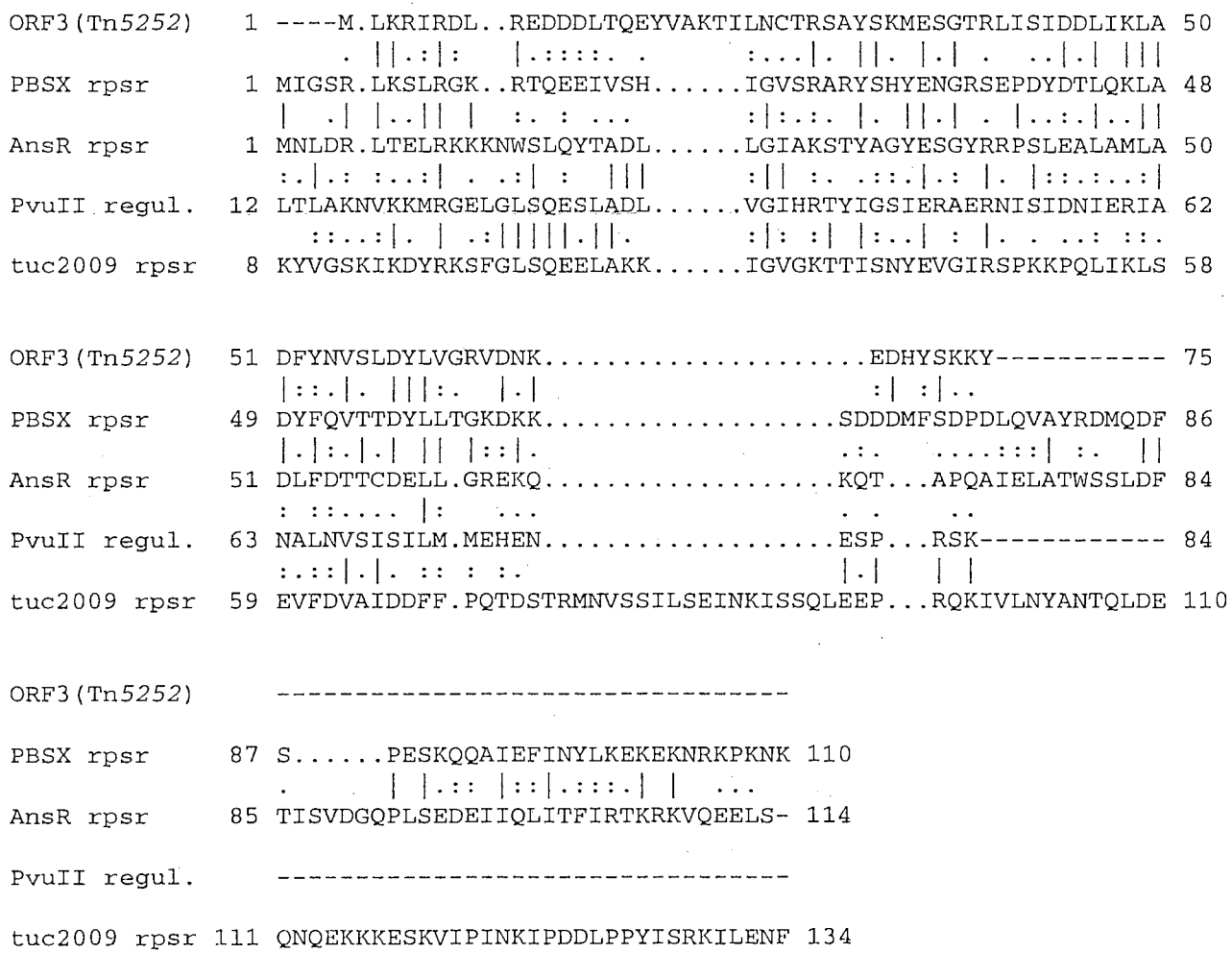


Figure 6. Alignment of ORF3 of Tn*5252* with repressors and regulators of different systems. Bars indicate identity and dots show conserved substitutions. The alignment was prepared by using the BESTFIT program in the GCG software package (University of Wisconsin).

similarity) (57). Figure 7 represents the alignment between the deduced amino acid sequence of ORF5 with that of the excisionase of Tn*5276*.

Insertion mutagenesis of the ORF3 locus

The homology between the predicted protein product of ORF3 and the transcriptional repressors of other systems suggested that this protein could also function in a similar fashion to the *c*I repressor of the coli-phage λ (42). If such is the case, upon mutating the ORF3 locus, we would expect a high frequency of excision of the transposon. To test this hypothesis, we created an insertion mutation using the *E. coli* plasmid pVA891 to interrupt ORF3. pVA891 is not capable of autonomous replication in streptococci, but expresses erythromycin upon insertion into the host chromosome. The strategy for creating insertion/deletion mutations within Tn*5252* has been described in detail elsewhere (38). The basic strategy, in brief, was as follows: DNA restriction fragments were cloned into pUC-based vector plasmids (such as pUC8 and pBluescript) and recombinant plasmids were generated in the *E. coli* strain JM109 by transformation (31). The passenger DNA in the recombinant plasmid contained a unique restriction site which was used to linearize it. Subsequently, a DNA segment carrying a drug resistance marker (pVA891) was ligated to the linearized plasmid. The resulting hybrid plasmid, a circular molecule, was digested with another enzyme that cleaved only the vector portion. The linear DNA molecule was used as donor DNA in transformation experiments using SP1000 as the recipient (Rx1 is our standard laboratory strain (69), and SP1000 is Rx1 carrying Tn*5252* and *str-1* chromosomal point mutation). The heterologous drug resistance marker is inserted into the recipient genome using the homology provided by the flanking donor DNA for synapsis and genetic integration. An added advantage with this method is that it does not result in any duplication of the directing DNA. Figure 8 gives a schematic representation of the insertion mutagenesis strategy.

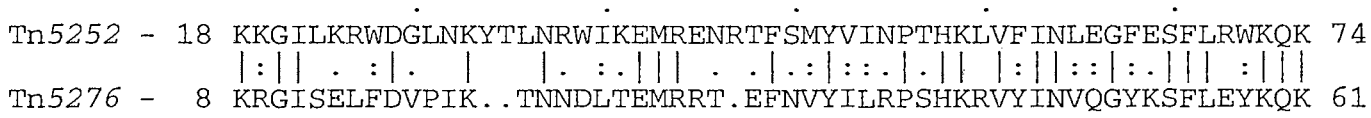
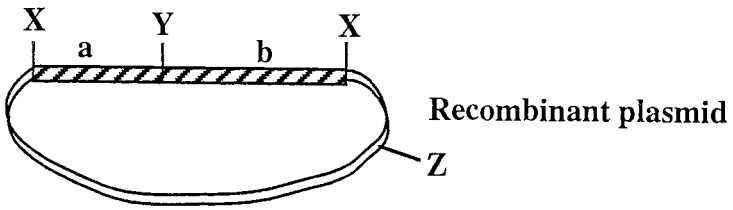
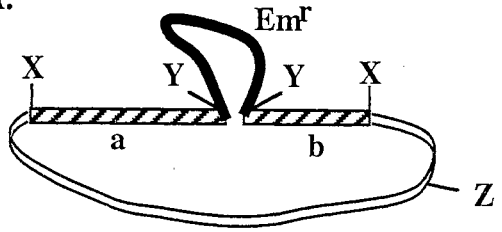


Figure 7. Similarity between the deduced amino acid sequence of ORF5 and the excisionase protein of conjugative transposon *Tn5276* found in *L. lactis* . Bars indicate identity and dots show conserved substitutions. The alignment was prepared by using the BESTFIT program in the GCG software package (University of Wisconsin).

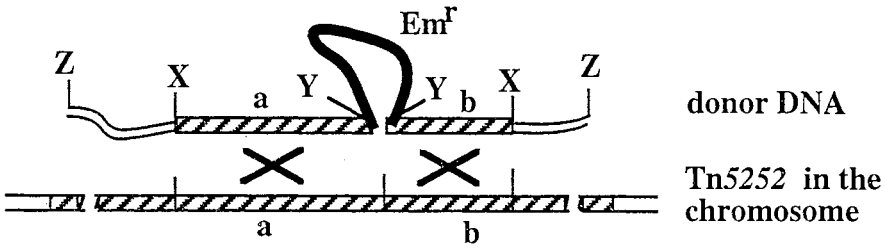
A - Isolate and ligate a fragment from the transposon to a suitable vector



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



C - Linearize the vector at the restriction site Z , and use it to transform SP1000 (Tn*5252*)



D - The resulting insertion mutant (Tn*5252* ::Em^r)

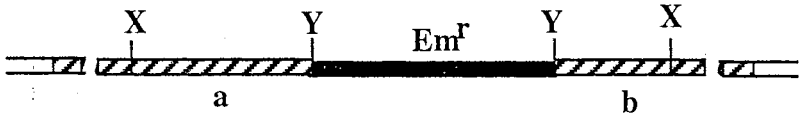


Figure. 8. Stratagy for creating insertion mutations within Tn*5252*. A DNA segment derived from the transposon and flanked by the restriction sites (X) is cloned into an *E. coli* vector plasmid. The recombinant plasmid is linearized upon digestion with a restriction enzyme that cleaves at the unique site (Y) and ligated to a marker DNA segment, Emr. The resulting circle is again linearized at a site (Z) within the vector plasmid. The linear DNA is used a donor DNA to transform pneumococcal cells carrying Tn*5252*, and the transformants are selected on plates containing erythromycin. (D) Expected product of such an event.

pSP111 was used to create a mutation within the putative repressor gene. However, the only available unique restriction site within ORF3 was *Kpn*I that was also present in the SK+ vector portion. To overcome this problem, the *Kpn*I site in the multiple cloning site of SK+ was destroyed by treatment with Klenow followed by self-ligation and the resulting vector was used to reclone the 1.07 kb *Xba*I fragment from pVJ15 to create the recombinant plasmid pSP143. The *Kpn*I site within the ORF3 region was used to linearize pSP143 and for insertion of pVA891. For the insertion of the heterologous DNA, the entire *E. coli* plasmid, pVA891, carrying resistance to chloramphenicol and erythromycin was used. However, pVA891 does not have *Kpn*I restriction site. Therefore, the *Kpn*I digest of pSP143 and the *Xba*I digest of pVA891 were digested with klenow fragment of DNA polymerase I. After both the molecules were ligated, the mixture was digested with *Pst*I to cleave the vector portion and this DNA was used to transform SP1000. The resulting Em^r transformants were screened for unselected markers, Cm^r, Amp^s and Str^r by replica plating. The three Em^r transformants obtained were used in the subsequent experiments.

Confirmation of insertion mutagenesis using pneumococcal transformation

To confirm the insertion of the vector plasmid within the transposon, transformation properties of the insertion mutants were studied. This experiment was conducted only with one of the mutant strains, designated SP1261. The rationale of this experiment was that if pVA891 had indeed integrated into the transposon in the mutant strain, and not in any other genomic location, then transformation frequency for the plasmid marker (Em^r) of a recipient strain bearing the element should be more efficient than transformation of the wild type strain (Rx1) because of the flanking homology provided by the donor DNA (38) in this homologous recombination event. Chromosomal DNA from one of the three Em^r transformants was isolated and used as donor DNA in transformation

of DP1322, bearing the element and the wild type, Rx1. As a control, the same two recipient strains were transformed with DP1617 DNA (Rx1 with *nov1* chromosomal point mutation) to nov^r transformants. The donor DNA was able to transform SP1000 cells three orders of magnitude higher than Rx1 cells to Em^r. On the other hand, the level of competence in both the strains was approximately equal as determined from the nov^r transformants resulting from transformation with DP1617 DNA. These results indicated that the *E. coli* plasmid pVA891 had indeed integrated at the intended site creating a mutant strain as expected. The results of this experiment are tabulated in Table V.

To confirm the above results we performed another transformation experiment. Genomic DNA from SP1261 (the mutant strain), and the genomic DNA from SP1254 (Tn5252 Ω pVA891, tra+) were used in this experiment. Sheared and unsheared DNA of the donor was used to differentiate between point and insertion mutations. Table VI represents the results of this transformation experiment. The recipients used were Rx1 and DP1322 (Rx1 carrying Tn*5252*). The reason for using sheared DNA was to show that genetic exchange of a fragment of DNA carrying a point mutation would be expected to be at least 10 to 100 fold higher than the one carrying 6 kb (pVA891) of heterologous DNA. No such increase in genetic exchange was noted. The results obtained were consistent with our earlier interpretation.

Southern Hybridization to Demonstrate the Insertion of pVA891 in SP1261

Hybridization using pSP111 carrying ORF3 as the probe

All three mutant clones were further used to physically confirm the insertion of pVA891 by Southern hybridization. The chromosomal DNA from SP1261 was digested with *Eco*RI and *Hin*dIII, fragments separated by electrophoresis on 0.8% agarose gels and transferred to nylon membranes. Chromosomal DNA isolated from Rx1 and SP1000

TABLE V

TRANSFORMATION FREQUENCY OF ORF3 MUTANT (SP1261)

DONOR DNA	RECIPIENT	SELECTION	CFU/ML
SP1261	SP1000	Em_3	58,000
SP1261	Rx1	Em_3	50
DP1617	SP1000	nov_{10}	60,000
DP1617	Rx1	nov_{10}	$>10^6$

TABLE VI

TRANSFORMATION FREQUENCY OF SHEARED VS. UNSHEARED DNA OF ORF-3 MUTANT AND SP1254

DONOR DNA	RECIPIENT	SELECTION	CFU/ML
SHEARED			
SP1261	Rx1	str_{200}	230,000
SP1261	Rx1	Em_3	35
SP1261	DP1322	str_{200}	73,000
SP1261	DP1322	Em_3	12,000
SP1254	Rx1	str_{200}	440,000
SP1254	Rx1	Em_3	<10
SP1254	DP1322	str_{200}	250,000
SP1254	DP1322	Em_3	230
UNSHEARED			
SP1261	Rx1	str_{200}	490,000
SP1261	Rx1	Em_3	60
SP1261	DP1322	str_{200}	54,000
SP1261	DP1322	Em_3	6,100
SP1254	Rx1	str_{200}	300,000
SP1254	Rx1	Em_3	<10
SP1254	DP1322	str_{200}	190,000
SP1254	DP1322	Em_3	300

digested with the same enzymes served as controls. The blotted DNA digests were probed with radiolabeled pSP111 DNA, washed and exposed to X-ray film. The autoradiogram of one experiment is shown in Figure 9. As evident from the autoradiogram, pSP111 did not hybridize with the Rx1 DNA as expected while it hybridized to a 1.07 kb *Eco*RI fragment and a >10 kb *Hin*dIII fragment of SP1000 DNA. With the insertion mutants, the probe was expected to hybridize with two *Eco*RI fragments of 1.98 and 5.0 kb and two *Hin*dIII fragments of 5.26 kb and 9.4 kb if a simple insertion of pVA891 had taken place within ORF3 (74). However, the hybridization data indicate that insertion of pVA891 at this site resulted in deletion of 2.5 kb DNA at the right end of the inserted DNA resulting in DNA fragments of varying sizes hybridizing to the probe. It had been observed before that when pVA891 is used for creating insertion mutants, it leads to varying lengths of deletions (unpublished data). For further analysis, only one of the mutants, designated SP1261 was used.

Verification of the deletion in pVA891

To more precisely locate the deleted segment of DNA in pVA891, Southern hybridizations were performed using different sets of digested DNA and probes. Rx1, SP1000, and SP1261 DNAs were digested with *Cla*I and *Bgl*II. As reference, the *Eco*RI and *Hind*III digested DNAs of SP1000 and Rx1 were also run on the same gel and blotted on nylon membranes. The probes used were pSP118 carrying the 0.6 kb DNA (coordinates 3.5-4.1 kb) and pSP119 carrying the 0.5 kb DNA (coordinates 4.1-4.6 kb). Hybridization with these probes was expected to show whether the deletion included the transposon DNA. Since the insertion was directed into the *Kpn*I site in the middle of the 1.07 kb *Xba*I fragment, it separated the 1.07 kb DNA into 0.6 and 0.5 kb DNA fragments to the left and the right of the insertion of pVA891, respectively. Both the probes hybridized with the DNA from SP1261 indicating that the deletion did not include the

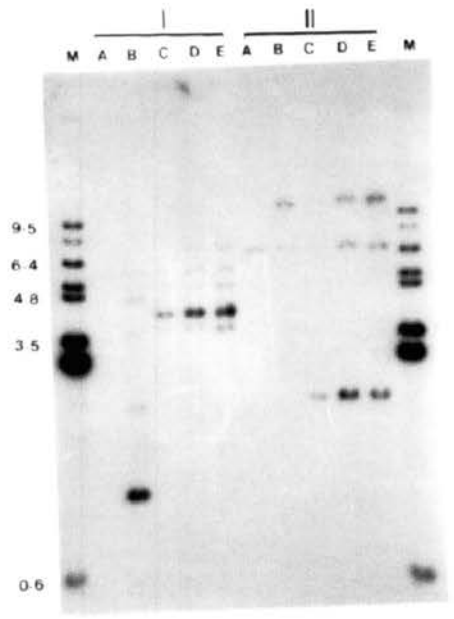


Figure 9. Physical analysis of Em^r transformants carrying the insertion of pVA891 within Tn*5252*. Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pSP111 to DNA from (A) Rx1, (B) SP1000, and (C-E) three Em^r transformants. *Eco*RI digests of DNA are shown in panel I and *Hind*III digests in panel II. Lanes M, marker DNA.

transposon DNA (Figures 10 and 11). Based on the hybridization pattern for the different DNA digests, it was evident that the deletion spanned the origin of replication and the chloramphenicol resistance gene of pVA891 and did not include the transposon DNA. This experiment showed that the transposon DNA in the immediate vicinity of the insertion was intact (Figure 12). Therefore the mutant SP1261 was used for further studies.

Circular Intermediate

Our working hypothesis was that ORF3 may act in a manner similar to the *cI* repressor of λ phage. If true, introduction of an insertion mutation in ORF3 should lead to loss of function of the putative repressor gene product. Hence, the transposon would not be under the control of this regulatory mechanism, and may therefore excise at a higher frequency from the host genome. If such an event takes place, we should be able to detect the excised transposon. To test this, we carried out Southern hybridizations using the transposon's target sequence and the right and left junction fragments as probes. pVJ187 is an *E. coli* plasmid carrying the target region of Rx1 for the integration of Tn*5252* (2.1 kb *Eco*RI fragment containing the target region, unpublished data). The right junction probe used was pSJ107 carrying the 3.27 *Eco*RI fragment from the right terminus of the transposon cloned in pLS1 plasmid (unpublished data). The probe used for the left junction was pVJ414 containing 3.6 kb of DNA from the Rx1 genome and the left junction of the transposon. The DNAs from SP1261, Rx1 and SP1000 were digested with *Eco*RI and *Hind*III, electrophoresed on agarose gels, and transferred to nylon membranes. Each membrane was probed with each of the probes separately and exposed to X-ray films. If the transposon had excised it would have led to the regeneration of the target region (2.1 kb *Eco*RI and >10.0 kb *Hind*III) in the chromosome which would hybridize with pVJ187 (Figure 13). Excision of the transposon would generate a circular intermediate leading to the fusion of left junction (Figure 14) and right junction (Figure 15) forming a fusion

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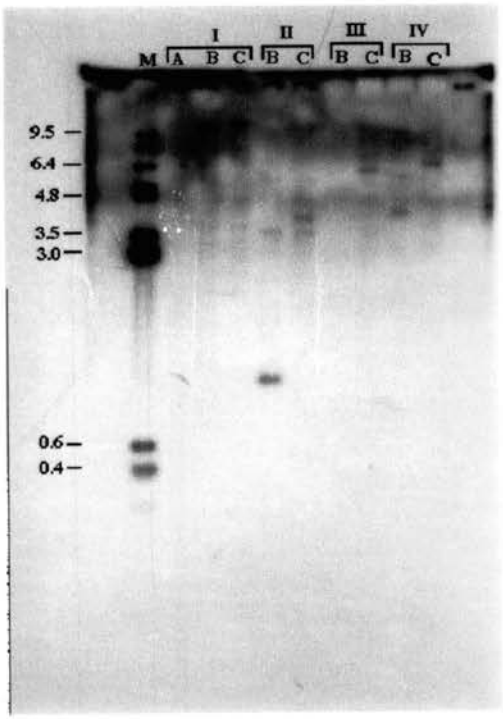

Figure 10. Verification of the extent of the deletion in SP1261 (ORF3 insertion mutant). Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pSP118 to DNA from (A) Rx1, (B) SP1000, and (C) SP1261. *Hind*III digests of DNA are shown in panel I; *Eco*RI digests in panel II; *Cla*I digests in panel III; and *Bgl*II digests in panel IV. Lane M, marker DNA.

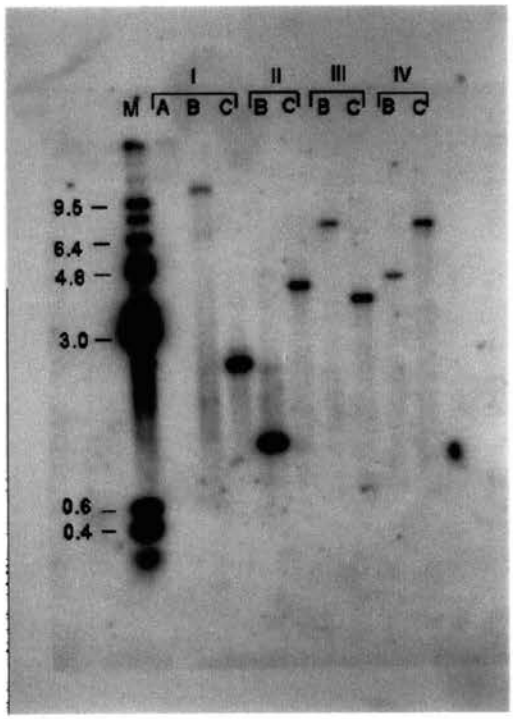

Figure 11. Verification of the extent of the deletion in SP1261 (ORF3 insertion mutant). Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pSP119 to DNA from (A) Rx1, (B) SP1000, and (C) SP1261. *Hind*III digests of DNA are shown in panel I; *Eco*RI digests in panel II; *Cla*I digests in panel III; and *Bgl*II digests in panel IV. Lane M, marker DNA.

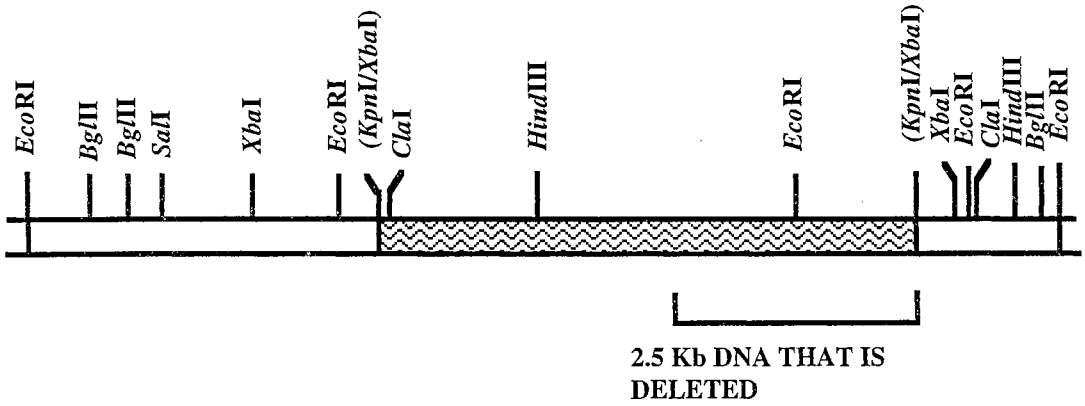

Figure 12. Restriction endonuclease map of SP1261 (ORF3 :: pVA891) showing the 2.5 kb deletion in the inserted DNA. Shaded area represents pVA891 DNA.

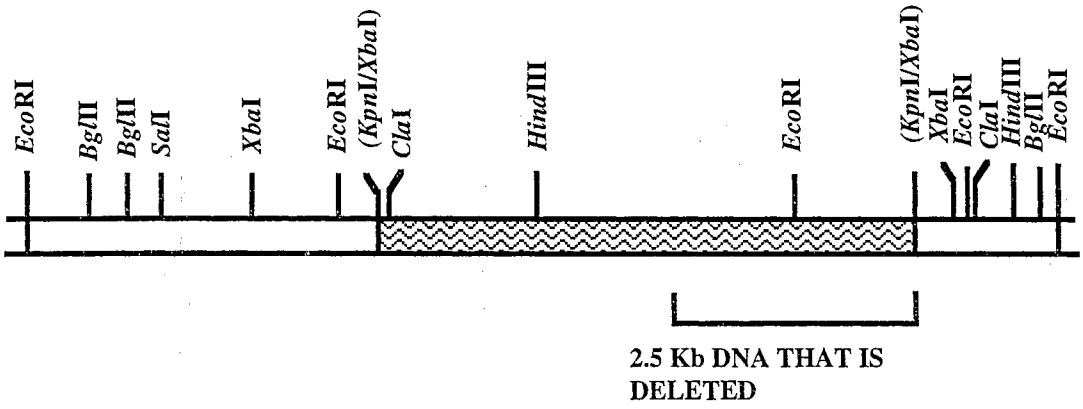

Figure 12. Restriction endonuclease map of SP1261 (ORF3 :: pVA891) showing the 2.5 kb deletion in the inserted DNA. Shaded area represents pVA891 DNA.

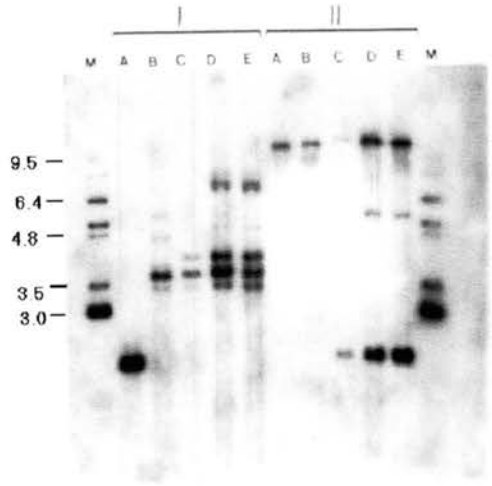

Figure 13. **Physical analysis of Em^r transformants (ORF3 insertion mutant) to detect a Circular Intermediate.** Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pVJ187 to DNA from (A) Rx1, (B) SP1000, and (C-E) three Em^r transformants. *Eco*RI digests of DNA are shown in panel I and *Hind*III digests in panel II. Lanes M, marker DNA.

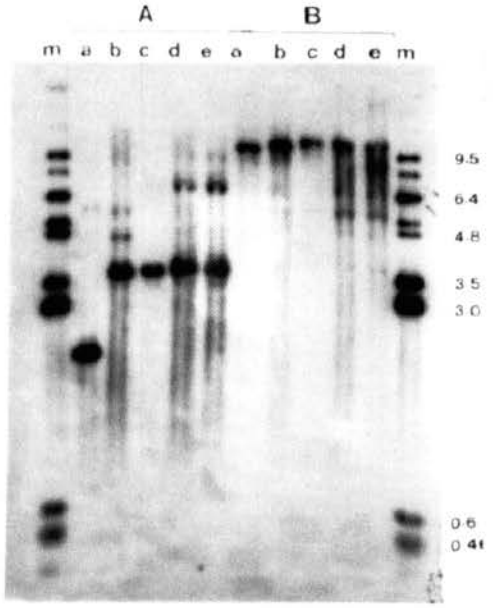

Figure 14. Physical analysis of Em^r transformants (ORF3 insertion mutant) to detect a Circular Intermediate. Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pVJ414 to DNA from (a) Rx1, (b) SP1000, and (c-e) three Em^r transformants. *Eco*RI digests of DNA are shown in panel A and *Hind*III digests in panel B. Lanes m, marker DNA.

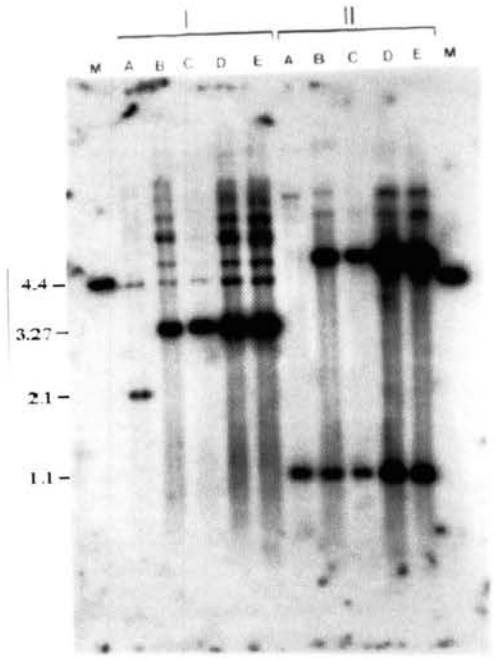

Figure 15. Physical analysis of Em^r transformants (ORF3 insertion mutant) to detect a Circular Intermediate. Autoradiogram showing DNA-DNA hybridization of ^{32}P-labeled pSJ107 to DNA from (A) Rx1, (B) SP1000, and (C-E) three Em^r transformants. *Eco*RI digests of DNA are shown in panel I and *Hind*III digests in panel II. Lanes M, marker DNA.

fragment (4.7 kb *Eco*RI and 6.5 kb *Hind*III). No bands close to the predicted size of a fusion fragment of the transposon ends or to the size of a regenerated Rx1 target region were observed. The results from these experiments implied that neither excision and circularization of the element nor "curing" of the cell took place in the mutant strains (74). The observed results were inconsistent with our hypothesis that the putative gene product of ORF3 functioned as the *E. coli* phage λ repressor.

Conjugation

Pneumococcus-Pneumococcus Conjugation

SP1261 was used in filter mating experiments to study the effect of the insertion mutation within ORF3 on the conjugal properties of the element from one pneumococcal strain to another. The donor and recipient cells were mated on a filter for 4 hours. Each mating experiment was performed in triplicate. After mating, the filters were washed separately, the cells in the supernatant of each of the three were pooled, and plated using the drug overlay method to score for transconjugants. Transconjugants were selected on plates containing erythromycin (transposon marker from the donor) and novobiocin (recipient chromosomal marker). The Em^r transconjugants were replica plated to screen for unselected markers, chloramphenicol (transposon marker) and streptomycin (donor chromosomal marker) to eliminate transformants. As a control, SP1254 (Tn*5252*ΩEm^r, tra+) (38) was used as a donor strain in conjugation. DP1002 (Rx1, nov^r), a pneumococcal strain that does not carry the element was used as the recipient. It has been shown earlier that the conjugal transfer frequencies of transfer proficient insertion mutants (such as SP1254) ranges from 10^{-7} to 10^{-6} per donor cell (38). In this study, the transfer frequency of SP1254 ranged from 10^{-7} to 10^{-5}. The conjugal transfer frequency of the

mutant strain, SP1261, was found to be less than 10^{-9} to 10^{-8} per donor cell. Results of the effect of the mutation on the conjugal transfer of the element is summarized in Table VII. In every case there is at least a 100 to 1000-fold reduction in the transfer of the element from SP1261 as compared to the control strain SP1254, thus implying that ORF3 is required for efficient conjugal transfer of the element (74).

Pneumococcus to *S. pyogenes* conjugation

Conjugation experiments were also performed using *S. pyogenes* strains ATCC19615 and ATCC21547 as recipients. Filter matings were done for 4 h. The harvested cells were plated for transconjugants on selective agar plates by spread plating. The transconjugants were selected for erythromycin (donor marker) and optochin (recipient marker). The Em^r transconjugants were further replica plated on blood agar plates with the unselected antibiotics, chloramphenicol (transposon marker) and streptomycin (donor marker). Here again the transfer was found to be impaired in the mutant strain, SP1261, when compared with SP1254, the tra+ insertion strain. The results of these experiments tabulated in Table VII confirmed that the product of ORF3 was involved in the intercellular transfer of Tn*5252*.

Complementation

Construction of pSP149

SP1261 was observed to be transfer deficient. To test whether the ORF3 mutation in SP1261 could be restored by complementation in trans, we constructed a recombinant streptococcal plasmid carrying ORF3. The streptococcal plasmid pLS1 is a derivative of pMV158 (9) and is a useful vector for cloning genes in *S. pneumoniae* (76).

TABLE VII

EFFECT OF THE ORF3 MUTATION ON THE CONJUGAL TANSFER PROPERTIES OF Tn*5252*

DONOR	RECIPIENT	SELECTION	TRANSCONJUGANTS PER DONOR
SP1254	DP1002	Em, *nov*	2.2×10^{-6}
SP1261	DP1002	Em, *nov*	$< 1 \times 10^{-9}$
SP1254	SY21547	Em, *opt*	1.5×10^{-6}
SP1261	SY21547	Em, *opt*	$< 1 \times 10^{-9}$
SP1254	SY19615	Em, *opt*	1.3×10^{-5}
SP1261	SY19615	Em, *opt*	1.6×10^{-7}

Filter matings were done for 4 hours at 37°C.
SY represents *Streptococcus pyogenes*.
DP1002 is *Streptococcus pneumoniae* (Rx1) that is *nov*^{r}.

pLS1 has the ability to replicate and confer tetracycline resistance in both Gram-positive and Gram-negative bacteria. A copy number mutation of pLS1 doubled the plasmid copy number to 48 and 8, in *S. pneumoniae* and *E. coli* respectively (40). Replication of streptococcal plasmids in *E. coli* require functional *pol*A and *rec*A genes. Therefore, for the generation of recombinant plasmids constructed using pLS1, the *rec* proficient C600 strain of *E. coli* was used. The vector pLS1 was digested with *Eco*RI for cloning the 1.0 kb *Eco*RI transposon DNA fragment containing ORFs 3, 7, 8, and 31. A part of the multiple cloning site of Bluescript SK+ was also present in the 1.0 kb passenger DNA. Also, *Eco*RI digestion deletes the carboxy terminus of ORF5, the excisionase gene. pSP111 DNA was digested with *Eco*RI and the 1.0 kb fragment was separated on agarose gel and electroeluted. This fragment was ligated to *Eco*RI digested pLS1, transformed into competent C600 cells, and plated on LB agar plates containing tetracycline. Tetracycline resistant transformants were isolated and checked for the presence of the recombinant plasmid, designated pSP149. The plasmid, pSP149 was isolated by alkaline-SDS lysis, followed by CsCl density gradient centrifugation. Using appropriate restriction enzymes the orientation of the passenger DNA in pSP149 was determined. This was further confirmed by sequencing the plasmid using M13F primer as the primer binding site is present on the insert DNA.

Transformation of pSP149

pSP149 was transformed into SP1261 and SP1254. The Tc^r colonies were grown in CATPG broth without selection. The rapid lysis miniprep was performed to check for the presence of the plasmid. SP1261 and SP1254 carrying the plasmid pSP149 were used as donors. As a control for this set of experiments, pLS1, was also transformed into SP1261 as well as the transfer proficient insertion strain, SP1254.

Effect of pLS1 on the conjugal transfer properties of Tn*5252*

Filter mating experiments were performed as described earlier using the following strains as donors: SP1254 (Tn*5252*ΩEm^r, tra+), SP1254 (pLS1), SP1261, and SP1261 (pLS1). The recipient strain used was DP1002 (Rx1, nov^r). The transconjugants were selected for Em^r and Nov^r. The Em^r colonies were further replica plated for unselected markers (Cm, str, and Tc). Table VIII summarizes the results obtained from these experiments. SP1254 retained its transfer properties as well as its efficiency of transfer ranging from 10^{-7} to 10^{-6}. On the other hand, SP1261 did not regain transfer function. These results indicate that the vector pLS1 does not affect the transfer of the element from one host to another.

Effect of pSP149 on the transfer of Tn*5252*

Complementation experiments were carried out with pSP149 to determine if the transfer properties could be regained upon introduction of a fragment of DNA carrying ORF3 on a multi copy plasmid. Filter matings were done as described earlier. The strains used as donors were SP1254, SP1254 (pSP149), SP1261, and SP1261 (pSP149). Pneumococcal strains DP1002 and DP1002 (pSP149) were the two recipients used for this set of matings. DP1002 containing pSP149 was also used as one of the recipient strains to check if the presence of the protein product in multiple copies in the recipient had any effect on the entry and integration of the element. We also wanted to determine the effect the protein had on the transfer of the wild type transposon. Hence we used SP1254 (pSP149) as one of the donors. A control mating was also performed using SP1254 as the donor and DP1002 as the recipient. The results of this experiment are tabulated in Table IX. As expected, the conjugal transfer frequency of the control mating was observed to be 10^{-7} to 10^{-6}. It was obvious from the complementation results that in spite of the presence of the

TABLE VIII

EFFECT OF pLS1 ON THE CONJUGAL TANSFER PROPERTIES OF Tn*5252*

DONOR	RECIPIENT	SELECTION	TRANSCONJUGANTS PER DONOR
SP1254 (pLS1)	DP1002	Em, *nov*	6.8×10^{-7}
SP1261 (pLS1)	DP1002	Em, *nov*	5.0×10^{-9}
SP1254	DP1002 (pLS1)	Em, *nov*	8.2×10^{-7}
SP1261	DP1002 (pLS1)	Em, *nov*	1.35×10^{-9}

TABLE IX

EFFECT OF pSP149 ON THE CONJUGAL TRANSFER PROPERTIES OF Tn*5252*

DONOR	RECIPIENT	SELECTION	TRANSCONJUGANTS PER DONOR
SP1254	DP1002	Em, *nov*	1.3×10^{-6}
SP1254 (pSP149)	DP1002	Em, *nov*	7.5×10^{-9}
SP1261 (pSP149)	DP1002	Em, *nov*	$<1 \times 10^{-9}$
SP1254	DP1002 (pSP149)	Em, *nov*	5.0×10^{-7}
SP1261	DP1002 (pSP149)	Em, *nov*	$<1 \times 10^{-9}$

plasmid pSP149 in the mutant, SP1261 did not regain transfer functions. The presence of the plasmid in the recipient strain also gave similar results. The element carrying a mutation in ORF3 in SP1261 was still transfer-deficient indicating that the transfer function was not restored even when an intact copy of ORF3 was present in trans. However, it was possible that the other ORFs also present in the passenger DNA could have affected the normal functioning of the ORF3 protein product.

The most interesting observation in this set of matings was the effect of the plasmid (pSP149) on the transfer of the element from the transfer proficient strain SP1254. When pSP149 was present in this strain it seemed to have an adverse effect on the transfer of Tn*5252*. Transfer was reduced by 1000 fold as opposed to the control. Curiously, the 1 kb fragment cloned into the plasmid also seemed to play some role in the entry or integrative functions of the element. When SP1254 was mated with a DP1002 strain containing the plasmid pSP149, there was a 10 fold reduction in the transfer frequency of the element. The reason could be the presence of the ORF3 protein in multiple copies in the strain. Presence of a regulatory protein in excess in a transfer proficient strain such as SP1254 may be inhibiting the normal transfer frequency of the transposon, perhaps by regulating other transfer related genes. The presence of other ORFs in the 1 kb region could also be a reason for the observed effect. Therefore any of the ORFs present in this 1 kb of DNA could be responsible for the loss of transfer functions in SP1254.

One of the factors that needs to be considered in this type of mating is the presence of other open reading frames besides ORF3 in the 1.0 kb fragment cloned for creating pSP149. There is a total of 4 intact open reading frames in this fragment. This might lead to problems during interpretation of results because at this juncture we do not really know the importance of the other open reading frames. ORFs 7 and 31 did not show sufficient homology to any known proteins in the databank, did not contain strong promoter sequences, and are transcribed in the opposite direction from that of ORF3 and ORF8. Therefore the possibility that ORF31 and ORF7 may have any role in the conjugal transfer

was presumed unlikely. It is possible that ORF3 or ORF8 may contribute to the observed effect.

Construction of pSP152

To determine the ORFs involved in the abolition of transfer of a normally transfer proficient strain such as SP1254, we used pSP149 to create a mutant of ORF3. The unique *Kpn*I site present in ORF3 was the site chosen to introduce the mutation. pSP149 was digested with *Kpn*I, and the exonuclease activity of the T4 DNA polymerase on single stranded DNA was utilized to remove the 3' overhang, followed by ligation. The DNA was transformed into C600, a rec+ host *E. coli* competent cells. Tetracycline resistant transformants were selected. Plasmid DNAs from two Tc^r transformants were isolated, digested with *Kpn*I, and run on an agarose gel to verify loss of the *Kpn*I site. As shown in Figure 16 both clones showed the loss of the *Kpn*I site. pSP149, the parent plasmid and pBluescript SK+ plasmid were used as positive controls. The plasmids were also digested with *Hind*III to verify the quality of the DNA used. The loss of the *Kpn*I site was evident from the migration of the DNA samples. DNA sequencing of one of the mutants designated pSP152 using primers close to the *Kpn*I site on either side showed that it had a 4 base pair deletion at the *Kpn*I site and the rest of the transposon DNA was intact upstream and downstream of the deletion. pSP152 was used in complementation studies.

Effect of pSP152 on the conjugal transfer of Tn*5252*

pSP152 was transformed into SP1254, SP1261 and DP1002 to yield Tc^r transformants. Plasmid minipreps were done to verify the presence of the plasmid in these strains. For filter matings, the donors were SP1254, SP1254 (pSP152), SP1261, and

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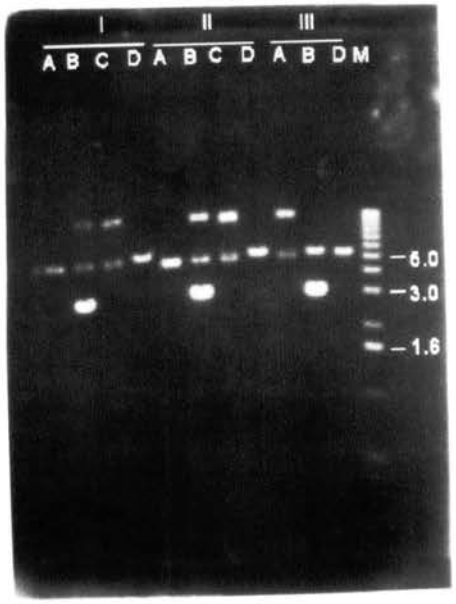


Figure 16. Verification of the Mutation at the *Kpn*I site in pSP149. Panel I represents the DNA samples of one of the clones designated pSP150, panel II-pSP152, and panel III-pSP149. (A) undigested DNA sample; (B) *Kpn*I digested DNA mixture containing the clone DNA and SK+; (C) *Kpn*I digest of the clone; and (D) *Hind*III digest of the clone.

SP1261 (pSP152). The recipients were DP1002 and DP1002 (pSP152). When SP1261 was used as the donor with or without the plasmid, no transconjugants were obtained as seen previously (Table X). This suggested that for the conjugal transfer of the element, ORF3 protein seemed to be important and that without a functional copy of this gene in cis, the transposon was unable to mobilize. Similar results were observed when the matings were done using *S. pyogenes* ATCC 21547 as the recipient.

In the earlier section we discussed the effect of pSP149 (which had 4 ORFs intact) on the transfer of Tn*5252* from SP1254. The presence of the plasmid in a normally transfer proficient strain like SP1254 seemed to abolish transfer functions. When pSP152 was present in SP1254, it still behaved in the same manner, indicating that ORFs other than ORF3 may be responsible for the observed effect. Therefore, it was crucial to perform experiments to answer questions such as: Will ORF3 alone in trans allow the transposon to regain its transfer functions? Which of the ORFs contributes to the abolition of transfer in SP1254? We sought to answer these questions by performing the experiments described below.

Construction of pSP153, pSP154 and pSP156

To answer the above questions it was important to clone one ORF at a time in pLS1 and perform filter mating experiments. To construct a plasmid containing only ORF3 in pLS1, a DNA fragment containing ORF3 and its putative promoter sequence was amplified by PCR. The primers were designed in such a way that one of the primers contained an *Eco*RI site and the other primer the *Hin*dIII site. This facilitated "force-cloning" of the *Eco*RI/*Hind*III digested amplified fragment into *Eco*RI/*Hind*III digested pLS1. ORF3-F2 primer, (5'-GAAAA**GAATTC**ACATTTAGAGTAACAGTTTCC-3') had the *Eco*RI site and ORF3-R2 (5'-CCAT**AAGCTT**CTGCTCCACAAATGAGG-3') was the reverse primer containing the *Hind*III site. The bold letters in the primers used for PCR indicate

TABLE X

EFFECT OF pSP152 ON THE CONJUGAL TRANSFER PROPERTIES OF Tn*5252*

DONOR	RECIPIENT	SELECTION	TRANSCONJUGANTS PER DONOR
SP1254	SY21547	Em, *opt*	1.5×10^{-6}
SP1261 (pSP152)	SY21547	Em, *opt*	$<1 \times 10^{-9}$
SP1254	DP1002	Em, *nov*	1.3×10^{-6}
SP1254 (pSP152)	DP1002	Em, *nov*	$<1 \times 10^{-9}$
SP1261 (pSP152)	DP1002	Em, *nov*	$<1 \times 10^{-9}$
SP1261	DP1002 (pSP152)	Em, *nov*	$<1 \times 10^{-9}$

the restriction endonuclease site incorporated into the primers. The template DNA used was pSP111 linearized with *Sac*I. The PCR products were double digested with *Eco*RI and *Hind*III so as to have compatible ends with similarly digested vector. The digested DNA was used in ligations with pLS1 double digested with *Eco*RI and *Hind*III. Double digestion of pLS1 would lead to loss of 150 base pairs of pLS1 DNA. This loss does not affect any of the functions of pLS1 as a vector. After ligation, the DNA was used in transformation of *E. coli* C600 and Tc^r colonies were scored for. Plasmid minipreps of six colonies were made to verify the presence of the plasmid. One of the DNA samples was digested with *Eco*RI and *Hind*III to confirm the integrity of the clone. This recombinant plasmid carrying ORF3 was called pSP153.

Similarly, ORF7 and ORF8 were cloned by PCR. The primers used for ORF7 were ORF7R (5'-GACCTTATC**AAGCTT**GCAGA-3') and M13R primer, and the primers used for ORF8 were ORF8-R primer, (5'-TATA**AAGCTT**TTAGGAAACTGTTACTC -3') and KS+ primer. The template DNA used in both the cases was pSP111 linearized with *Sac*I. The recombinant plasmid carrying ORF7 was called pSP154 and ORF8 was pSP156.

The plasmids were transformed into the different strains of pneumococci depending on the type of matings to be performed. Filter matings were done using these strains as the donors and DP1002 as the recipient. As a control, SP1254 was mated with DP1002.

Effect of pSP153 (ORF3) on the Conjugal Transfer of Tn*5252*

SP1261 (pSP153) and SP1254 (pSP153) as donors and DP1002 as recipient were used for this mating experiment. Transconjugants were selected for Em^r and nov^r markers and replica plated for unselected markers Cm^r, and str^s. The results of this mating are tabulated in Table XI. Tn*5252* from SP1254 donor cells was able to transfer at a frequency of 10^{-5} transconjugants per donor as expected and SP1261 showed a transfer frequency of

TABLE XI

EFFECT OF pSP153 and pSP154 ON THE CONJUGAL TRANSFER PROPERTIES OF Tn*5252*

DONOR	RECIPIENT	SELECTION	TRANSCONJUGANTS PER DONOR
SP1254	DP1002	Em, *nov*	1.4×10^{-5}
SP1261	DP1002	Em, *nov*	2.4×10^{-7}
SP1254 (pSP154)	DP1002	Em, *nov*	7.2×10^{-6}
SP1254 (pSP153)	DP1002	Em, *nov*	59×10^{-9}
SP1261 (pSP153)	DP1002	Em, *nov*	$< 1 \times 10^{-9}$

* pSP154 has the entire ORF7 in the vector pLS1. The insert 667 bp, long was amplified using M13R primer and ORF7R primer (location 670-651 in the sequence given). pSP153 has the entire ORF3 in the vector pLS1. The 404 bp insert was amplified using ORF3F2 (location 404-426) and ORF3R2 (location 792-808 in the sequence given).

10^{-7}. As observed before, there is a 100-fold difference in the transfer frequency of the transposon in these donors. However, SP1261 (pSP153: ORF3) did not regain transfer function. In fact, there is a 100-fold drop in the frequency of transfer of Tn*5252* from this donor to DP1002. This indicated to us that ORF3 cannot be complemented when present in trans. Moreover, it seemed to be regulating other genes which are important in transfer of the element as evidenced from the fact that the presence of multiple copies of this gene product lowered the transfer frequency in wild type cells as well as the mutant strain. It will be interesting to see the effect a single copy of this product in trans has on the transfer of the element.

Effect of pSP154 (ORF7) on the Conjugal Transfer of Tn*5252*

Presence of pSP149 (pLS1 carrying the 1.0 kb EcoRI fragment) or pSP152 (pSP149 with ORF3 mutation) in the normally transfer proficient strain SP1254, leads to a loss of transfer of the element to DP1002. To test if ORF7 played a part in this observation, we conducted these set of matings using SP1254, and SP1254 (pSP154) as donors. SP1254 transferred the element with the expected frequency (Table XI). However, when the plasmid containing ORF7 was present in the donor, the transfer efficiency dropped only by five-fold. This result indicated that ORF7 is probably not the gene responsible for the earlier result, as the drop in transfer frequency was not as dramatic as when the entire 1.0 kb insert was provided in trans on a multicopy plasmid.

Effect of pSP156 (ORF8) on the conjugal transfer of Tn*5252*

pSP156 was transformed into SP1261, SP1254 and DP1002. The resultant transformants were checked for the presence of the plasmid. The donors were SP1261, SP1261 (pSP156), SP1254 and SP1254 (pSP156) which served as the control. The

recipients used were DP1002 and DP1002 (pSP156). Filters matings were done as described earlier and the transconjugants were selected for nov^r and Em^r. The results of this experiment are tabulated in Table XII. SP1254 transferred the transposon to DP1002 as expected at a frequency of 10^{-5} transconjugants per donor. SP1261 was *tra-* as expected. The presence of pSP156 in SP1261 did not alter the transfer properties of SP1261, reiterating that ORF3 is important for the conjugal transfer of Tn*5252*. Similar results were obtained with SP1261 mated with DP1002 carrying the plasmid, pSP156. However, when SP1254 was mated with DP1002 (pSP156), the transfer seemed to drop by an order of magnitude, an observation made earlier in the chapter when DP1002 (pSP149) was used as the recipient. Similarly, when SP1254 (pSP156) was mated with DP1002, it was observed that the transfer efficiency of Tn*5252* from SP1254 (pSP156) was at least five fold lower than that of SP1254 alone. It was interesting to note that transfer of the element was not completely abolished when ORF8 alone was present in SP1254. These results indicated that probably ORF8 had some role in the transfer of the element but definitely was not the gene responsible for the effect observed earlier with pSP149.

Protein Expression

The synthesis of a protein of correct size is a useful means of verifying the gene product of a particular DNA sequence. *In vivo* protein expression assays were carried out to identify protein products made from the 1.07 kb DNA under study and to verify which of the ORFs present in this region actually encode functional proteins.

TABLE XII

EFFECT OF pSP156 ON THE CONJUGAL TRANSFER PROPERTIES OF Tn*5252*

DONOR	RECIPIENT	SELECTION	TRANSCONJUGANTS PER DONOR
SP1254	DP1002	Em, *nov*	2.5×10^{-5}
SP1261	DP1002	Em, *nov*	5.7×10^{-9}
SP1254 (pSP156)	DP1002	Em, *nov*	7.0×10^{-6}
SP1261 (pSP156)	DP1002	Em, *nov*	2.9×10^{-9}
SP1254	DP1002 (pSP156)	Em, *nov*	1.1×10^{-6}
SP1261	DP1002 (pSP156)	Em, *nov*	2.3×10^{-9}
SP1261 (pSP156)	DP1002 (pSP156)	Em, *nov*	$<4.0 \times 10^{-9}$

*pSP156 has the entire ORF8 in the vector pLS1. The 374 bp insert was amplfied using KS^+ primer and ORF8R primer (location 431-412 in the sequence given) using pSP111 as the template.

^{35}S labeling of proteins after infection and induction by phage and IPTG

Radioactive labeling of plasmid-specific products was carried out as described in the methods section. This is a modification of the method described by Tabor et al. (79), requiring the passenger DNA be cloned in pBluescript plasmids in the right orientation such that the ORFs are under the control of the T7 promoter present on the vector portion of the recombinant plasmid. T7 RNA polymerase gene has been placed in M13 mGP1-2 phage under TAC promoter control and this could be induced with IPTG. Therefore cells containing the recombinant plasmids could express the genes carried on the insert DNA upon infection with M13 mGP1-2 phage and subsequent induction of the T7 RNA polymerase gene by IPTG.

pSP111, pSP118, pSP119 and pSP151 were the plasmids used for this study. All of these are pBluescript plasmids carrying the 1.07 kb DNA under study in its entirety in either orientations, or as deletions spanning different regions (Figure 17). pBluescript SK+ served as the control. SDS-PAGE analysis of the radiolabeled proteins synthesized in *E. coli* cells containing plasmids carrying varying segments of DNA derived from the 1.07 kb region enabled us to identify the protein products of ORF3 and ORF8 (Figure 18). Two polypeptides, each about 9 kDa were synthesized in cells harboring pSP111 (lane C) carrying the 1.07 kb *Xba*I fragment that contains ORF3 and ORF8 in proper orientation downstream of the T7 promoter. The observed sizes of the products of ORF3 and ORF8 were in reasonable agreement with their estimated sizes based on deduced amino acid sequences. However, these disappeared in cells harboring pSP118 (lane D) that carried a deletion of 0.5 kb *Xba*I/*Kpn*I DNA segment containing ORF8 and the amino terminal of ORF3. A polypeptide of about 9 kDa corresponding to the predicted product of ORF8 was observed in the extracts of cells with the plasmid, pSP119 (lane E), carrying this 0.6 kb *Xba*I/*Kpn*I DNA segment and the deletion of the rest from this region. On the other hand, cells carrying pSP151 (lane B) that contains the same passenger fragment as in pSP111 but

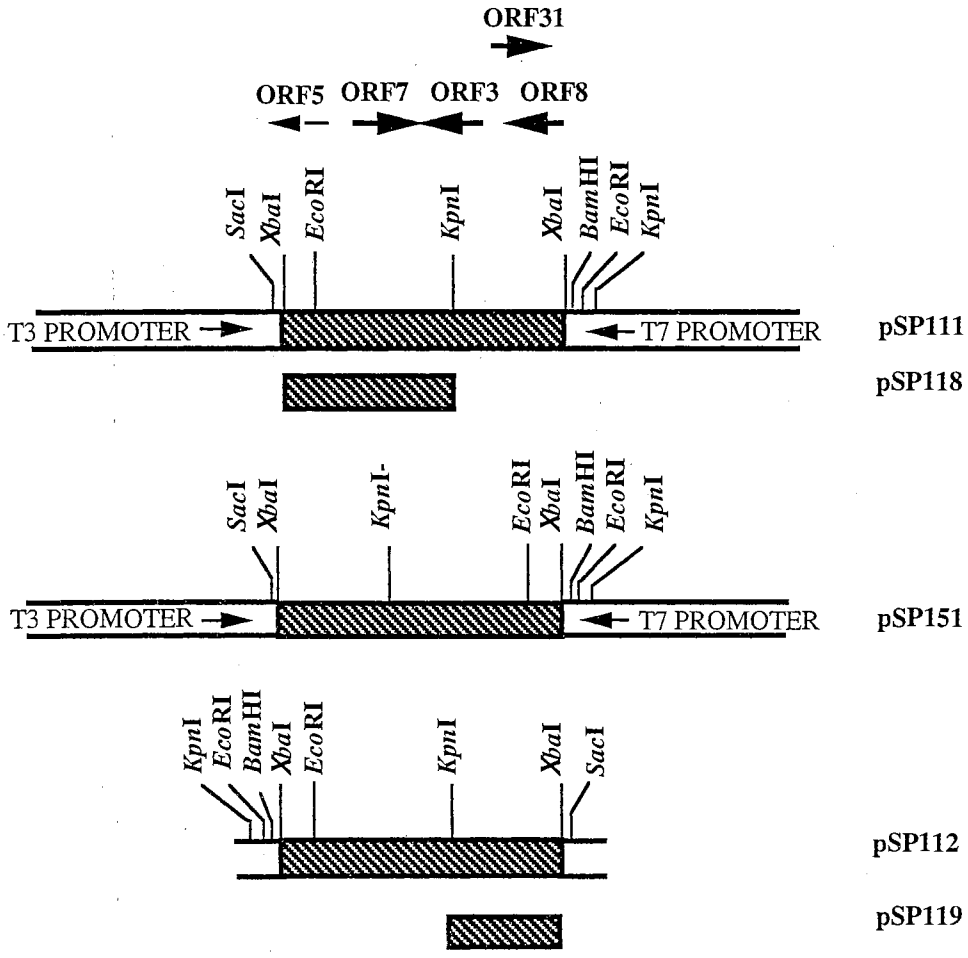


Figure 17. Map of pSP111, pSP151, pSP112 and their deletion derivatives. Shaded area represents the 1.07 kb *Xba*I fragment of the left terminus of Tn*5252*. The passenger DNAs in various plasmids are shown above.

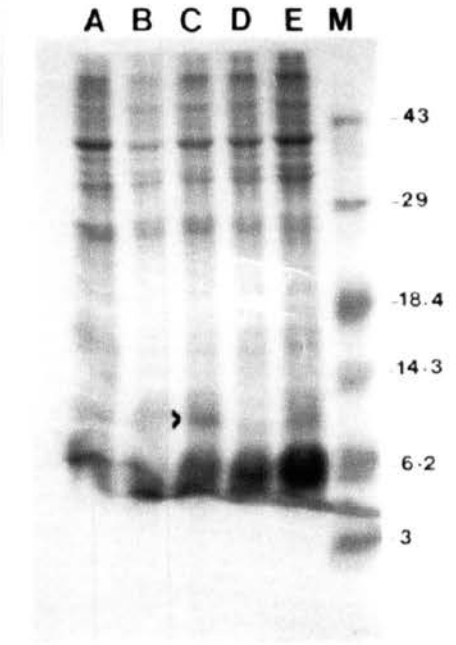


Figure 18. Autoradiogram showing SDS-PAGE profiles of protein products of ORF3 and ORF8. (^{35}S)methionine and (^{3}H)leucine labeled extracts of *E. coli* cells carrying various plasmids induced with 1 mM IPTG were fractionated on a 17% (w/v) discontinuous polyacrylamide gel. The radioactive polypeptides were detected using fluorography. Profiles shown correspond to *E. coli* cells harboring pBS-SK+ (lane A), pSP151 (lane B), pSP111 (lane C), pSP118 (lane D), and pSP119 (lane E). The arrow indicates the two comigrating polypeptides specified by the passenger DNA in pSP111. The molecular weights (in kDa) of ^{14}C-labeled standard proteins are shown on the right (lane M).

in the opposite orientation, no plasmid specific product was visible. The 5.7 kDa protein expected from ORF7, however, may not be resolved in the gel system used. ORF31, the other open reading frame that could be transcribed in this plasmid by the T7 polymerase, also did not seem to make the corresponding protein product. The reason could be the absence of a good promoter sequence upstream of the start site for ORF31. We did not expect the protein product of ORF5 in this study at all, because the carboxy terminus of this open reading frame is absent in the clones used in this experiment. This experiment demonstrated that the 1.07 kb region under investigation in this study produced at least two protein products that could be detected in the gel system used. It is evident from the sizes of the proteins observed, that these were the products of ORF3 and ORF8 genes (74).

Expression and Purification of Proteins from Cloned Genes

The ORF3 protein product was expressed and purified in large quantities to be used in DNA binding and footprinting assays.

Polymerase Chain Reaction of ORF3

The primers used were 5'-ATGTTGAAAAGGATTAGAGA-3', the forward primer and 5'-AATCATCTCCTAATA-3', the reverse primer. The forward primer begins at the start site of ORF3 gene and the reverse primer is 968 bp downstream of the stop site of ORF3 gene. The template used was 4.4 kb *Bgl*II fragment isolated from the plasmid, pVJ15.

Cloning into pMAL-p2

The 1137 bp PCR fragment was cloned into the *E. coli* plasmid, pMAL-p2 vector at the *Xmn*I site. It is designed to create fusions between a cloned gene and the *E. coli malE* gene, which codes for the maltose binding protein (MBP). The MBP fusion can then be expressed and purified, taking advantage of the properties of MBP. The method uses the strong "tac" promoter and the *malE* translation initiation signals to give high-level expression of the cloned sequences and a one step purification of the fusion protein using MBP's affinity for maltose. The signal peptide on the p2 vector directs the fusion protein to the periplasm. The *lacI*^{q} gene which codes for the *lac* repressor keeps expression from the P_{tac} low in the absence of IPTG induction. The pMAL vector also contains the sequence coding for the recognition site of the specific protease Factor Xa located just 5' to the polylinker insertion site. This allows MBP to be cleaved from the protein of interest after purification with few or no vector derived residues attached to the protein of interest.

The PCR fragment was cloned in to the *Xmn*I site of the pMAL-p2 vector. The inserts were verified for their integrity by sequencing using the *malE* primer and the M13 sequencing primers. One recombinant plasmid was named pSP155.

Pilot Study

A pilot experiment was set up to determine the behavior of the MBP fusion protein (50 kDa) from pSP155. The following samples were made during this experiment: uninduced and induced cells, a total cell crude extract, a suspension of the insoluble material from the crude extract, a fraction containing protein that binds to the amylose resin, and a periplasmic fraction prepared by the cold osmotic shock procedure. The protein samples from the pilot study were run on a 17% SDS-PAGE gel and used to determine the location of the protein of interest and the time course of highest expression level. The gel

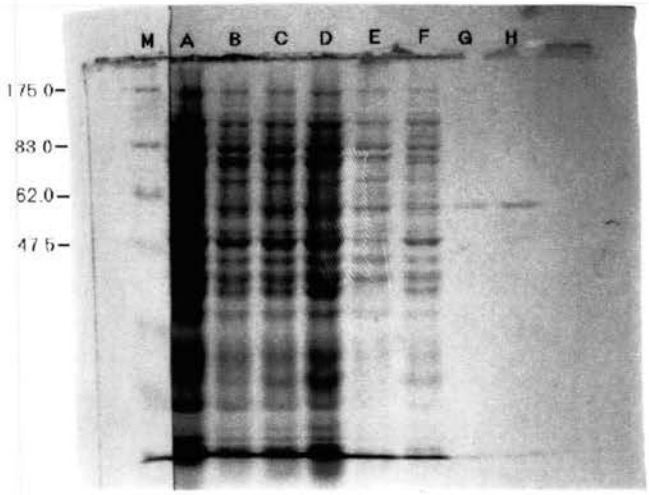


Figure 19. Pilot Experiment to Optimize Expression of ORF3 product from pSP155 (pMAL-p2::ORF3). Coomassie stained 12% (w/v) SDS-PAGE gel. The lanes represent M. prestained protein marker, B. 1 hr induction sample, C. 2 hr induction sample, D. 3 hr induction sample, E. crude extract, F. insoluble matter, G. amylose bound sample, and H. sample after cold osmotic shock.

picture (Figure 19) showed that the protein was present in the soluble fraction as well as the periplasmic fraction and its expression was highest at 3 h after induction.

Protein Purification

The plasmid, pSP155 was used for purification of the fusion protein from 1 liter culture by affinity chromatography as described in the methods section. Fourteen fractions were collected and a sample of each fraction was assayed for protein concentration by the Bradford method as described in the methods section. Fractions containing the highest amount of proteins were pooled. A pilot Factor Xa digestion was set up with 20 μg of the protein and 0.2 mg of factor Xa followed by an incubation at 37°C, for 12 h. The 50 kDa fusion protein was resistant to cleavage possibly because the 42 kDa MBP masked the cleavage site (Figure 20). Therefore for DNA binding studies, the fusion protein was used instead of the pure protein free of MBP.

DNA Binding Assay using MBP Fusion Protein

Homology search of ORF 3 showed significant homologies with regulatory proteins. Regulatory proteins function by binding to specific DNA sequences in the vicinity of the genes they regulate. When a protein binds the DNA, it retards the migration of the protein-DNA complex, which could be visualized as a shift in the gel. We sought to determine the binding sites for this protein using this concept. Therefore we set up DNA binding assays using linearized DNA from the left junction containing the various ORFs we believed could be the target(s) for this protein. The DNA used were pVJ429 (0.1-3.6), pSP110 (2.8-3.5), pSP111 (3.5-4.6), and pSP108 (4.6-8.0). The coordinates of the

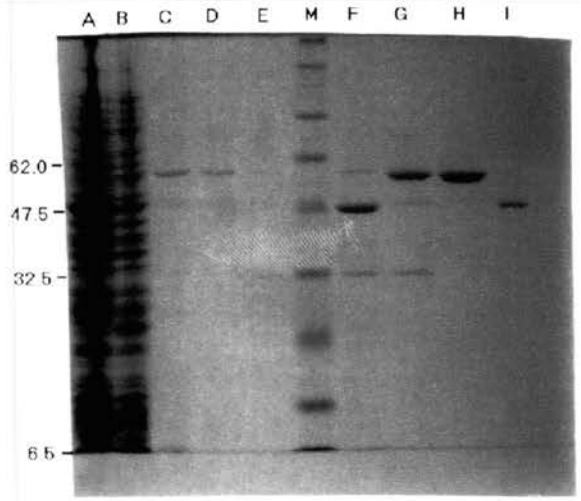


Figure 20. Large Scale Protein Purification from pSP155 (pMAL-p2::ORF3). 12% (w/v) SDS-PAGE gel after Coomassie staining. The lanes correspond to A. uninduced sample, B. sample after 3 hr induction, C. sample after cold osmotic shock, D. protein bound to amylose resin, E. amylose bound fusion protein mixed with maltose and Factor Xa, M. prestained protein marker, F. fusion protein mixed with Factor Xa, G. fusion protein mixed with Factor Xa and Ca Cl_2, H. fusion protein, and I. maltose binding protein.

DNA with respect to the transposon is given in the brackets. 0 represents the left junction of Tn*5252* (Figure 2).

A typical reaction consisted of 2 μl linearized DNA, 0.5 μg of fusion protein (50μg/ml), 10 mM Tris-HCl, pH 7.4, 0.1 mM EDTA, 1 mM DTT, and 25 mM NaCl in a 15 μl reaction volume. The reaction was incubated at 37°C for 30 minutes and immediately loaded on a 0.8% agarose gel in 1/2X TBE. The gel was stained with ethidium bromide and visualized under an UV illuminator (Figure 21). The slow migration of pSP110 showed DNA binding activity, and pSP111 also showed some activity. The rest of the DNA samples did not show any binding activity. pVJ429 contains 3.5 kb *Eco*RI fragment which also includes the 0.7 kb XbaI fragment cloned in pSP110. However, there was no shift in the migration of pVJ429 DNA. The reason could be that the fusion MBP protein probably masks the binding site and therefore binding is very poor in the reactions.

The protein yield from the pMAL system was very low, and the 42 kDa protein, MBP, could not be cleaved away from the small target protein by factor Xa. Also, the DNA binding assays using the MBP-ORF3 fusion protein were not efficient. Therefore we adopted a different expression system, the pET system (Novagen), for large scale protein purification.

Cloning ORF3 in pET-30b(+) Protein Expression Vector

Target genes cloned in the pET plasmids are under the control of strong bacteriophage T7 transcription signals. Expression is induced by providing a source of T7 RNA polymerase in the host cell. T7 polymerase is so active that almost all of the cell machinery is used for production of the protein under its control. This results in the desired product representing up to 50% of the total cell protein within a few hours of induction.

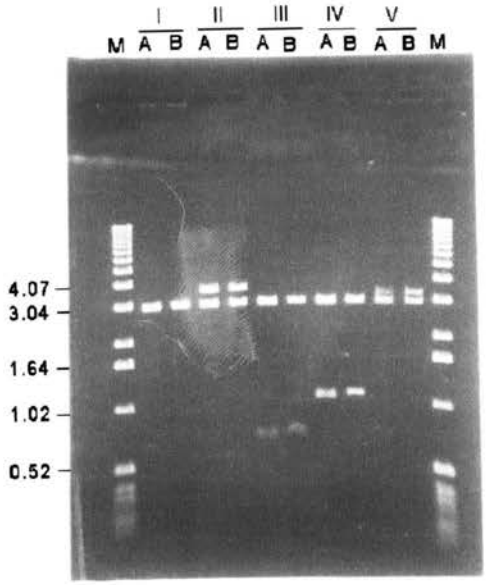


Figure 21. DNA Binding Assay using the Protein Product of ORF3. Lane M represents the 1 kb molecular ladder, Lanes I through V represent SK+, pVJ429, pSP110, pSP111, and pSP108, respectively. The letter A represents the DNA sample without the protein as a control for each type of DNA and B represents the reaction with protein.

This system also allows for the maintenance of potentially lethal target genes in the uninduced state since target genes are initially cloned in hosts that do not contain the T7 polymerase gene. Plasmids are then transferred into the expression hosts containing a chromosomal copy of the T7 RNA polymerase gene under *lac*UV5 control, and IPTG is used to induce expression. The pET vectors also have the advantage of carrying the His.Tag sequence, a stretch of 6-10 consecutive histidine residues which bind to the divalent cation (Ni^{2+}) immobilized on the His.Bind metal chelation resin. Thus, affinity chromatography can be performed to separate the protein of interest from the rest of the proteins.

In this study we used the pET-30a-c(+) vectors. The a-c denotes the reading frame relative to the *Bam*HI cloning site. Therefore, vectors having the suffix a express from the GGA triplet, the suffix b from the GAT triplet and the letter c from the ATC triplet of the GGATCC *Bam*HI recognition sequence. The vector chosen for cloning was pET-30b, because cloning a fragment isolated from pSP155 into this vector would have the right reading frame for expression of the protein from the fragment of interest.

Construction of pSP157

The pET-30b(+) vector was digested with *Eco*RV followed by *Hind*III. pSP155, the recombinant clone containing the ORF3 PCR product in the pMAL-p2 vector, was digested with *Ecl*136II followed by *Hind*III which would release the fragment of interest. This fragment also included the Factor Xa cleavage site which is necessary for separating the protein from the fusion. The 1.1 kb fragment was electroeluted in a 0.8% agarose gel, extracted with phenol:chloroform, and the DNA precipitated in 95% ethanol. This fragment was ligated to pET-30b, the DNA was used to transform *E. coli* JM109 competent cells, and cells plated on kanamycin $_{70}$ LB agar plates. Since blue/white

screening was not available, colonies were randomly picked and inoculated for minipreps to screen for a clone carrying the insert. Four clones were identified.

To confirm these constructs, two were sequenced using T7 promoter primer which is upstream of the start codon. One of the clones which had the proper sequence was designated pSP157. This plasmid was purified from *E. coli* JM109 and transferred to host *E. coli* strain containing a chromosomal copy of the gene for T7 RNA polymerase. We used *E. coli* BL21(DE3) cells, which are the most widely used hosts for target gene expression from pET vectors. This is a lysogen for the bacteriophage DE3, a lambda derivative that has the immunity region of phage 21 and carries a DNA fragment containing the *lac*I gene, the *lac*UV5 promoter, and the gene for the T7 RNA polymerase. The immunity region is inserted at the *int* gene of DE3, thereby preventing the integration as well as excision of the phage without a helper phage. Target DNA is transcribed by the addition of IPTG which induces T7 RNA polymerase production which is under the control of *lac*UV5 promoter.

Pilot Experiment

The expression of target genes was assessed by analysis of total cell protein on an SDS-PAGE gel followed by Coomassie blue staining. The cells were grown to an OD_{600} of 0.5, the T7 *lac* promoter induced by 1 mM IPTG, and the cultures allowed to grow for an additional 3 h. At the end of each hour 0.5 ml cells were removed, pelleted and resuspended in SDS-PAGE sample buffer. An uninduced sample was similarly prepared. As a control, a strain containing the vector only was also included in this pilot. All the samples were boiled at 100°C for 5 minutes and loaded on a 17% (w/v) polyacrylamide gel. The amount of the material loaded depended on the cell density at the time of harvest. Therefore, the amount loaded was reduced sequentially starting from the uninduced sample. It is evident from the gel (Figure 22) that pET-30b(+) does not make a protein of

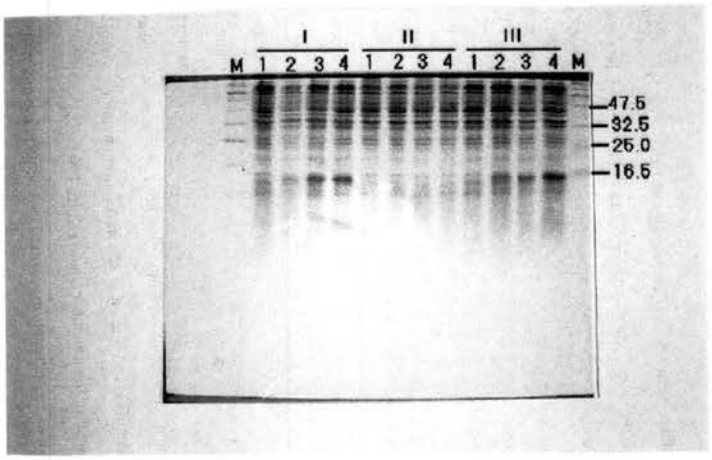


Figure 22. Pilot Experiment to Determine Time Course and Yield of ORF3 product using the pET system. The figure shows a 12% (w/v) SDS-PAGE gel stained with Coomassie Blue. The lanes represent M. molecular weight standard, 1. uninduced sample, 2. 1 hr post-induction sample, 3. 2 hr post-induction sample, and 4. 3 hr post-induction sample. Panel I corresponds to one of the clones #8, panel II to pET-30b (vector only), and panel III to clone #9. Clone #8 was designated pSP157 and used for large-scale purification.

the expected fusion protein size, about 16 kDa. However, it is very clear that a unique protein of 16 kDa is made by pSP157. The uninduced sample does not have this protein, but upon induction, the protein is expressed and the amount of the protein increases with increasing time. The optimal induction period was determined to be 2-3 h post induction for pSP157.

Purification of Target Proteins

E. coli BL21(DE3) cells carrying pSP157 were used for protein purification from a large culture. Fractions containing the protein were pooled together, dialyzed, and stored at -80°C for further studies.

Protease cleavage

The recombinant protein was cleaved with Factor Xa to remove the 64 amino acids upstream from the first methionine of the ORF3 protein. Factor Xa digestion was set up with 1 mg of the protein in a buffer containing 100 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1 mM $CaCl_2$ and 50 μg of Factor Xa. The incubation was carried out at 37°C for 18 hours. The cleavage products were passed over the chromatography resin a second time. The cleaved target protein was recovered in the flow through. However, the recovery of the unbound cleaved protein was not too high. Therefore the following experiments were performed using the uncleaved protein.

DNA Binding Assay

Dr. Vijayakumar did these experiments with the fusion protein I isolated from pSP157. The results obtained from these will be described in detail elsewhere. To show that the protein product of ORF3 is indeed a DNA binding protein and to show the region of binding, I have included the results of these experiments in this work. Based on the results obtained from Dr. Vijayakumar's DNA binding experiments, it was clear that the protein product of ORF3 was indeed a DNA-binding protein. It bound upstream of the start site of three open reading frames present in the left terminal region of Tn*5252*. Those ORFs include ORF5, ORF4 and ORF3. All of the above mentioned ORFs have been discussed in detail in this work. The predicted amino acid sequence of ORF5 has significant homology to the excisionase of a lactococcal transposon, Tn*5276*. ORF4 codes for the relaxase protein of Tn*5252*. All these observations lend support to the notion that the ORF3 product is a regulatory protein controlling more than one gene involved in the transfer of Tn*5252*. Also, it likely that ORF3 is autoregulatory since the ORF3 protein binds upstream of ORF3.

DISCUSSION

Because of the propensity of genetic elements such as Tn*5252* to autoaccumulate various drug resistance determinants and horizontally transfer themselves among a number of clinically relevant bacteria, an understanding of the transfer mechanism of these elements becomes important. A study of Tn*5252* could shed light on the biology of other complex composite streptococcal conjugative transposons due to a high degree of homology between
them (45). Previous work in our laboratory (38) seemed to indicate that the left end containing the chloramphenicol resistance gene may be involved in many of the transfer-

related functions of the element. In this study, we determined the DNA sequence and evaluated the genetic properties of a 1.07 kb DNA sequence adjacent to the integrase locus. Of the five ORFs, *in vivo* overexpression of recombinant plasmids containing DNA from this region led to the identification of the products of ORF3 and ORF8 on SDS-PAGE gels. Based on homology to peptide sequences in the data base, clues to the potential roles of the products of ORF5 and ORF3 were also obtained.

In an earlier paper, we showed that ORF2, located next to ORF5 and transcribed in the same orientation, could encode a polypeptide of 13.05 kDa (38). The 3'-end of this gene overlaps with the 5'-end of ORF1. Based on genetic studies showing that ORF2 carried a transfer-related function and the resemblance of the overall physical organization of this region to other site- specific recombination genes, we previously suggested that ORF2 was most likely the excisionase gene of Tn*5252* (38). However, the current results showing strong homology between ORF5 and the *xis* gene of the lactococcal conjugative transposon, Tn*5276* (57), seem to suggest that the organization of this region is more complicated than imagined.

The most significant finding in this study relates to the potential regulatory role of the region carrying ORF3. The homology displayed by the deduced amino acid sequence of ORF3 to transcriptional regulators of prokaryotic origin led us to examine the role of this region of DNA in the functioning of Tn*5252*. The decrease in transfer frequency when the donors or recipients carried pSP149 seems to indicate that the excision and integration activities are both subject to negative control by one or more products of the passenger DNA in this plasmid. However, the observation that the presence of pSP149 in the donors impedes the transfer frequency ten fold more than when in the recipients where only the integrase and not the excisionase activity is required indicates that the integrase is not subject to as stringent a control as that of the excisionase. It is worth pointing out that the motif AAAATA was found near the promoter region of ORF5 (suspected to encode the excisionase) and other promoter regions in the DNA segment described above but is not

found anywhere near the predicted transcription start site of ORF1 which encodes the integrase (38). It is likely that this site serves as a signal for the binding of the putative regulatory molecule. If the functional role of the predicted regulator was similar to one such as the cI repressor of phage, abolition of its function should lead to elevated levels of excision of the element from the chromosome. The mutation, however, lead to the loss of transfer of the transposon implying that the product of ORF3 was involved in transfer of Tn*5252* from one cell to another and that its activity was very different from that of cI repressor. The product of ORF3 cannot be provided in trans for regaining the function in the mutant strain. The presence of multiple copies of the ORF3 gene in a the wild-type host strain containing the transposon led to a drop in the transfer frequency by at least 100-fold. This implies that there is a concentration factor involved in the conjugal transfer of Tn*5252*. At least one functional copy of this protein must be present for the conjugal transfer of the element and the presence of multiple copies leads to a drop in the transfer frequency.

Our current view based on available information is that the coordinate derepression and induction of excision of Tn*5252* is dependent upon the receipt of a signal, possibly provided by cell-to-cell contact during mating. During this event, the excisionase and integrase genes are perhaps transcribed as a single unit from an inducible promoter. Upon entry into a recipient cell, integrase gene by itself could be transcribed from a different promoter by a host polymerase leading to the genetic integration of the element. In spite of the structural similarities between many of the protein products, the initial events and the regulatory circuits in conjugative transposons and temperate phages could possibly be different in subtle ways, reflecting uniquely adapted strategies to meet the survival needs of each. For example, excision upon simple derepression in the absence of a potential mate in close physical contact may not lead to, as in the case of phages, spread and survival of a conjugative transposon that is incapable of autonomous replication. If the term "immunity" refers to a detectable decrease in the frequency of superinfection and to a molecular switch that controls one life cycle over another, the experimental results presented above favor the

conclusion that the 1.07 kb DNA segment containing ORF3 could serve as the immunity region of Tn*5252*. Implicit in this hypothesis is the lack of a need for transfer of the integrase from the donor as suggested for the Tn*916*-type elements (7).

Even though the displayed homology indicates that the product of ORF3 is most likely the repressor molecule, more rigorous demonstration is needed. Definitive assignment of funtional roles for this gene and others in this region awaits future studies.

CHAPTER V

IDENTIFICATION OF A REGION ENCODING A DNA RELAXASE IN Tn*5252*

RESULTS AND DISCUSSION

Considerable attention has been given to the study of conjugative elements due to their capacity to autoaccumulate antibiotic resistance genes and disseminate them even among unrelated species of bacteria by a process that is DNase resistant and requires intimate contact on solid surfaces (15). The most studied conjugative transposon is Tn*916* for which the entire DNA sequence has been determined. However, a clear picture of the DNA processing mechanisms has not yet emerged in spite of the accumulated data. It is generally believed that conjugative tranposition is likely to be biochemically very distinct from the gram-negative plasmid conjugation systems as there is no requirement for pilin which is necessary for the recognition of potential recipient cells (86).

We have been investigating the genetics and biology of the pneumococcal conjugative transposon, Tn*5252*, carrying resistance to chloramphenicol (4). Besides the localization of several transfer-related regions (38), recent identification of a region adjacent to the integrase/excisionase gene which is responsible for transfer of the element, has led us to attempt identification of the region responsible for transfer.

It has been shown earlier that an insertion mutation in a *Bgl*II site (coordinate 5.5 kb) at the left terminus of the element led to a loss of transfer of the element from one cell to another (1). We sought to investigate the molecular details of this region in order to determine its biological role. Figure 2 shows the restriction map of Tn*5252* showing the 4.4 kb *Xba*I fragment under study highlighted.

Sequencing

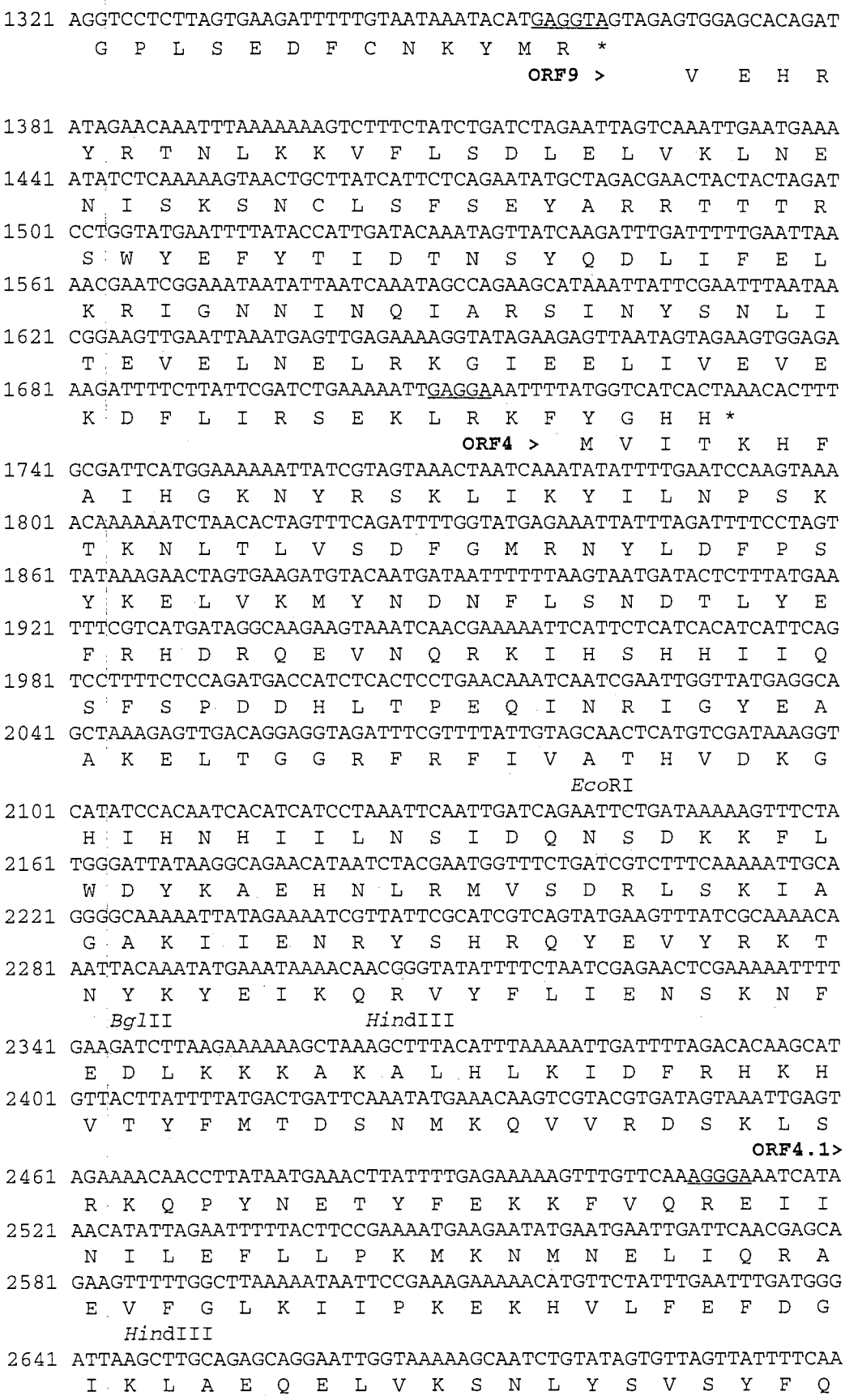
The 3.25 kb *Xba*I DNA fragment towards the left terminus of the element was the focus of this study. For sequencing, this fragment was cloned into Bluescript vectors. pVJ15 is an *E. coli* plasmid which carries about 10 kb of transposon DNA from the left terminus (unpublished data). This plasmid was digested with *Xba*I and the 3.25 kb *Xba*I fragment was isolated, subcloned into pBluescript SK+, and designated pSP108. Exonuclease III and S1 nuclease digestions were performed followed by self ligations to construct a nested set of deletion derivatives of pSP108 to be used for sequencing. Dideoxy nucleotide sequencing was performed as outlined in the methods section. Figure 3 shows the schematic representation of the strategy adopted for sequencing. Both double and single stranded DNA templates were used in sequencing reactions and, for maximal confidence, both strands were sequenced.

Sequence analysis

The sequenced region was found to be 3249 nt in length with a G+C content of 29.9%. Examination of the sequence translated in all six reading frames, revealed 5 open reading frames (ORFs), each with a potential initiation codon following an appropriate ribosome-binding site (RBS) (75). Curiously, there was an additional open reading frame (ORF4.1) within ORF4 with a potential RBS preceding the start codon, ATA. The translational start site for ORF4.1 was 266 codons downstream of the start site for ORF4. All six ORFs were located on the same strand and in the same orientation as shown in Figure 3. For easy visualization, the sequence data shown in Figure 23 is presented in the

*Hin*dIII
*Xba*I **IR1**
---------> <--------- -35
1 AAGCTTCTAGAACAATTGCTAGAAGCAAACTCATTGGTGGTCATTCAGCTGGAGGATTAG
PORF12 -10
61 ATGGACTAAAATAATGAATGTTCTTATTTACCTTTTGAGTCTGCCCGAGTATATCAGGAT
ORF12 >
121 GTGGCATGGCTAAGTGGATGCTTCTTTTTATCAGAACATTCCAGTTCTCTTTGGGCAGAA
V A W L S G C F F L S E H S S S L W A E
181 AAAAATAAAGAAGATATCTCCATTTCCCTATCATTGGAAGAGAAAGTTCTATTTGTTCGT
K N K E D I S I S L S L E E K V L F V R
241 CAACTTTATACGAATGTATTCCCTGCAACTTTTGTTTTTAAGTTTTCTAATCAAAGAAAA
Q L Y T N V F P A T F V F K F S N Q R K
301 GTAGTATCAGGTATTGTTAAAGAGATTGGAAAAGAGTTTATATCTATAAAATCAGACACT
V V S G I V K E I G K E F I S I K S D T
361 GGTTTTCTTCGATTAAAATGGGAAGATATACTCGATATACAGATAGAAGGGGAGGAATTA
G F L R L K W E D I L D I Q I E G E E L
421 CATGAATCGTAAAGAATTATATGATGATAAATTACAGCTAGATTATTTTTCAGATTCTTA
H E S * **ORF11 >**
M N R K E L Y D D K L Q L D Y F S D S Y
481 TTTACAGTTTGAGTCAGATTTTTACAAGTATTCAGCTTTAGATATACCATTAACATTTAT
L Q F E S D F Y K Y S A L D I P L T F I
541 CACAGATGATATTTTACGCACAATGGCTATGTCTCAAAAACATTATTTTAAACTTAACAA
T D D I L R T M A M S Q K H Y F K L N K
601 AAGTAAATCTTTAGACGGTCGTGATCATTACTTTGTTTTTTCTATCAAGATGAACAAAGA
S K S L D G R D H Y F V F S I K M N K D
661 CAGTAGTGGTATTAGACAGTATGAATATCAGAGACATTGTTTTAATTTGTAAGAGTCCGA
S S G I R Q Y E Y Q R H C F N L *
IR2
--------> <--------
721 CAGGGCTCTTTTTTCTGTGATAATTTTATCAAAAAGTATTTGTTATACTTTTTTTAATTT
781 AGATTTATCTTGGGGGTTGGGGGGCGTGCCACCACATTTTCATATCGTTTGTTTTTTAGA
841 ACCGTGAGGTTTGAAATGGCGGCGATATGATTTTTGGGATATTGTGGACACAATATCTGA
-35 **P**ORF10
---------->
901 GCTCGCAAAGCCATACAAAAATGTTGAATCTATTTTGTAAAACGTACTGACAGTGTATGT

*Hin*dIII
IR3 -10
<----------
961 AAGCTTACATTGTCAGTACAAGTATCTATGAGAGGAGGATAAATCATATGAAAAGAAATAT
ORF10 > M K R N I
1021 ACGTAGTATTCGGAAACAATTTCGCTTAACAGAAACGGAAGAAAAACAAATACTAGATTT
R S I R K Q F R L T E T E E K Q I L D L
*Hin*dIII
1081 AATGAGAGAGAAAGGAGATGATAATTTTTCTGATTTTCTCCGTAAAAGCTTACTATTATC
M R E K G D D N F S D F L R K S L L L S
1141 TGATGGACAAAAACAGATGGAAAAATGGTTCAACCTTTGGAAAAAACAAAAGTTGGAACA
D G Q K Q M E K W F N L W K K Q K L E Q
1201 AATTAGTCGTGATGTTCATGAAGTATTCATAATTGCTAAAACAAATCATCAGGTTACTCA
I S R D V H E V F I I A K T N H Q V T H
1261 CGAACATGTTTCTATTTTGTTAACTTGTATTCAGGAATTAATTAAAGAGGTAGAAAAAAC
E H V S I L L T C I Q E L I K E V E K T


1321 AGGTCCTCTTAGTGAAGATTTTTGTAATAAATACATGAGGTAGTAGAGTGGAGCACAGAT
G P L S E D F C N K Y M R *
ORF9 > V E H R
1381 ATAGAACAAATTTAAAAAAAGTCTTTCTATCTGATCTAGAATTAGTCAAATTGAATGAAA
Y R T N L K K V F L S D L E L V K L N E
1441 ATATCTCAAAAAGTAACTGCTTATCATTCTCAGAATATGCTAGACGAACTACTACTAGAT
N I S K S N C L S F S E Y A R R T T T R
1501 CCTGGTATGAATTTTATACCATTGATACAAATAGTTATCAAGATTTGATTTTTGAATTAA
S W Y E F Y T I D T N S Y Q D L I F E L
1561 AACGAATCGGAAATAATATTAATCAAATAGCCAGAAGCATAAATTATTCGAATTTAATAA
K R I G N N I N Q I A R S I N Y S N L I
1621 CGGAAGTTGAATTAAATGAGTTGAGAAAAGGTATAGAAGAGTTAATAGTAGAAGTGGAGA
T E V E L N E L R K G I E E L I V E V E
1681 AAGATTTTCTTATTCGATCTGAAAAATTGAGGAAATTTTATGGTCATCACTAAACACTTT
K D F L I R S E K L R K F Y G H H *
ORF4 > M V I T K H F
1741 GCGATTCATGGAAAAAATTATCGTAGTAAACTAATCAAATATATTTTGAATCCAAGTAAA
A I H G K N Y R S K L I K Y I L N P S K
1801 ACAAAAAATCTAACACTAGTTTCAGATTTTGGTATGAGAAATTATTTAGATTTTCCTAGT
T K N L T L V S D F G M R N Y L D F P S
1861 TATAAAGAACTAGTGAAGATGTACAATGATAATTTTTTAAGTAATGATACTCTTTATGAA
Y K E L V K M Y N D N F L S N D T L Y E
1921 TTTCGTCATGATAGGCAAGAAGTAAATCAACGAAAAATTCATTCTCATCACATCATTCAG
F R H D R Q E V N Q R K I H S H H I I Q
1981 TCCTTTTCTCCAGATGACCATCTCACTCCTGAACAAATCAATCGAATTGGTTATGAGGCA
S F S P D D H L T P E Q I N R I G Y E A
2041 GCTAAAGAGTTGACAGGAGGTAGATTTCGTTTTATTGTAGCAACTCATGTCGATAAAGGT
A K E L T G G R F R F I V A T H V D K G
EcoRI
2101 CATATCCACAATCACATCATCCTAAATTCAATTGATCAGAATTCTGATAAAAAGTTTCTA
H I H N H I I L N S I D Q N S D K K F L
2161 TGGGATTATAAGGCAGAACATAATCTACGAATGGTTTCTGATCGTCTTTCAAAAATTGCA
W D Y K A E H N L R M V S D R L S K I A
2221 GGGGCAAAAATTATAGAAAATCGTTATTCGCATCGTCAGTATGAAGTTTATCGCAAAACA
G A K I I E N R Y S H R Q Y E V Y R K T
2281 AATTACAAATATGAAATAAAACAACGGGTATATTTTCTAATCGAGAACTCGAAAAATTTT
N Y K Y E I K Q R V Y F L I E N S K N F
BglII HindIII
2341 GAAGATCTTAAGAAAAAAGCTAAAGCTTTACATTTAAAAATTGATTTTAGACACAAGCAT
E D L K K K A K A L H L K I D F R H K H
2401 GTTACTTATTTTATGACTGATTCAAATATGAAACAAGTCGTACGTGATAGTAAATTGAGT
V T Y F M T D S N M K Q V V R D S K L S
ORF4.1>
2461 AGAAAACAACCTTATAATGAAACTTATTTTGAGAAAAAGTTTGTTCAAAGGGAAATCATA
R K Q P Y N E T Y F E K K F V Q R E I I
2521 AACATATTAGAATTTTTACTTCCGAAAATGAAGAATATGAATGAATTGATTCAACGAGCA
N I L E F L L P K M K N M N E L I Q R A
2581 GAAGTTTTTGGCTTAAAAATAATTCCGAAAGAAAAACATGTTCTATTTGAATTTGATGGG
E V F G L K I I P K E K H V L F E F D G
HindIII
2641 ATTAAGCTTGCAGAGCAGGAATTGGTAAAAAGCAATCTGTATAGTGTTAGTTATTTTCAA
I K L A E Q E L V K S N L Y S V S Y F Q

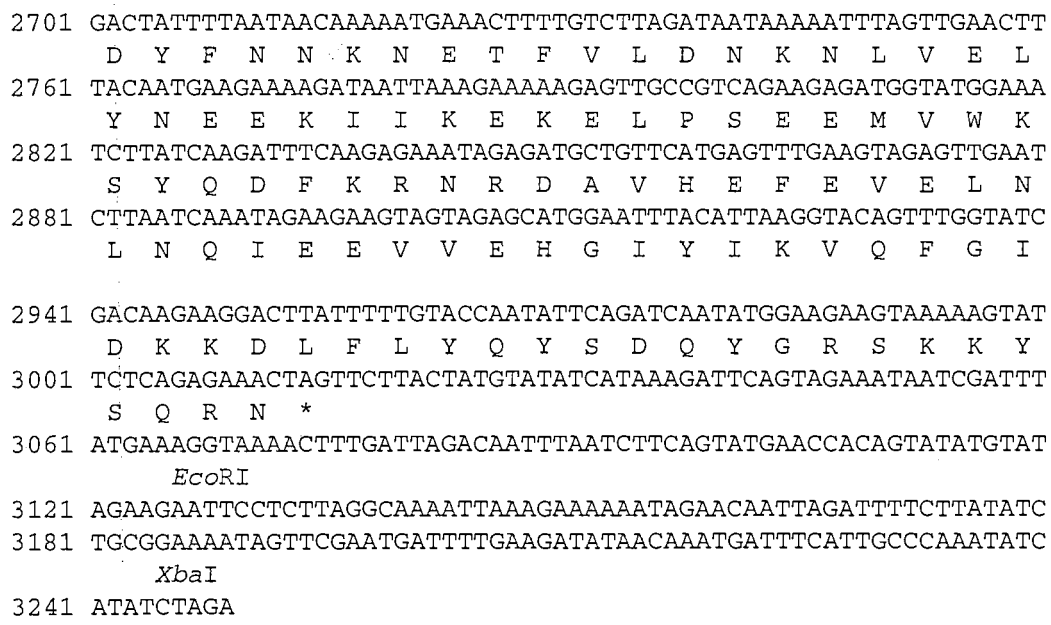


Figure 23. Nucleotide sequence of the transfer-related region encoding a putative DNA relaxase in Tn*5252*. The sequence begins from inside the element and reads towards the left terminus. Inverted repeat regions are indicated by arrows above the sequence. The potential -10 and -35 region are labeled. Putative ribosome-binding sites are underlined. Relevant restriction sites are given. Designated ORFs are specified at the beginnings of the corresponding amino acid sequence.

direction of transcription. The region was observed to be filled with ORFs, with the RBS of many cistrons overlapping with the adjacent ones, implying possible translational coupling. The structural properties of the deduced polypeptides is given in Table XIII.

Analysis of the noncoding regions indicated that these were probably involved in transcriptional regulation. On the basis of similarity to the consensus -10 and -35 motifs of gram-positive bacteria (28), a pair of promoters, P_{ORF12} and P_{ORF10}, were identified. P_{ORF12} could direct transcription of ORF12 and ORF11, while P_{ORF10} could direct transcription of ORF10, ORF9, ORF4, and ORF4.1. Regions carrying imperfect inverted repeats were associated with the two promoter sequences. The first, IR1, that could form a stem-loop structure with a G of -11.1 kcal/mole contained the motif CTAGAA directly repeated within itself. While the IR1 preceded the promoter region in P_{ORF12}, the -35 region was located within the regions of the inverted repeats, IR2 (G= -15.7 kcal/mole) in PORF10. Also there were shorter segments of dyad symmetry as well as directly repeated sequences within IR2. Examination of the sequence data also revealed a short stretch of GCs followed by several Us and a pair of inverted repeats, designated IR3, resembling a rho- independent terminator in the intergenic region between ORF12 and ORF10. Based on these observations, it would seem that there were two transcriptional units on this segment of DNA.

Sequence comparison

A GenBank search was undertaken to detect sequence similarities between the predicted products of ORFs and the protein sequences in the database. ORF9 was found to exhibit local homology to ORF1 (25% identity and 56% similarity) of the conjugative plasmid, pC1528, in *Lactococcus lactis* (46). The function of the predicted product of ORF1 in the conjugal transfer of pC1528 is yet to be determined. The deduced amino acid sequence of ORF4 was found to be significantly homologous to the predicted amino acid

TABLE XIII

PHYSICAL PROPERTIES OF PROTEIN PRODUCTS DEDUCED FROM THE DNA SEQUENCE IN FIGURE 23

ORF	MOLECULAR WEIGHT (kDa)	pI
4	52	9.1
4.1	19.9	5.2
9	14.5	8.3
10	14.2	9.0
11	11.7	7.2
12	11.9	4.9

sequence of the spliced *ltr*B ORF of the lactococcal plasmid, pRSO1 (49), and the Rep proteins encoded by mobilizable nonconjugative plasmids, pC223, pC221, and pT181 (21, 56) from *Staphylococcus aureus*. Among these, the highest level of homology was between the predicted product of ORF4 and DNA relaxation protein of *Staphylococcus aureus* plasmid, pC223 (28% identity, 51% similarity; Figure 24) (2, 75, 83). While the DNA relaxases do not share global amino acid similarity, all have been shown to carry three consensus motifs each representing a distinct functional domain. Replacement of specific and highly conserved amino acids in these motifs in the DNA relaxation protein of the conjugative plasmid, RP4, led to the identification of the catalytic roles associated with each of them (53) (Figure 24). The protein product of ORF4 was found to carry overall homology as well as in all three consensus motifs found in DNA relaxases suggesting functional similarity (75) (Figure 25). With other ORFs, no significant homologies were detected.

The 6.1 kb sequence of the left terminal region of Tn*5252* partly given in this work and elsewhere (38), did not show any consensus sequence that is usually associated with origins of transfer (52).

Protein Expression

In vitro transcription and translation

The *in vitro* transcription and translation assays were performed to determine the number and sizes of the protein products that could be made from the 3.25 kb DNA under study.

Figure 24. Similarity between the predicted amino acid sequence of ORF4 and the DNA relaxase of *S. aureus* plasmid pC223. Bars indicate identity and dots show conserved substitutions. Gaps were introduced to maximize similarity. The highly conserved amino acid residues in DNA relaxases are shown in boldface. The three motifs thought to be involved in site-specific binding and nicking of DNA are shown above the sequence.

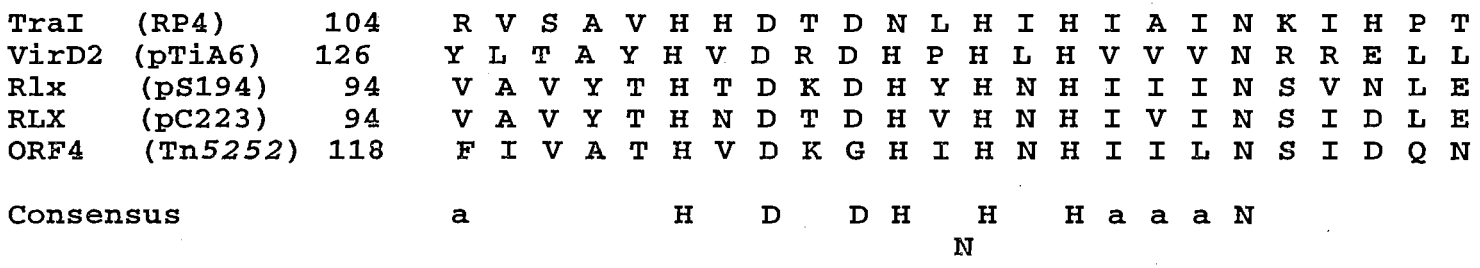


Figure 25. Alignment of the catalytic domain of relaxases of other systems with ORF4 of Tn*5252*.

E. coli S30 Extract System for Circular DNA

The *E. coli* S-30 system for in vitro transcription and translation of the ORFs under study was done as suggested by the manufacturer. A brief description of the system, procedure, and the reaction conditions are described in the methods section of this work. The plasmid DNAs used for this study were pSP108, pDR22, pLG151, pLG159, pLG148, and pLG156 (Figure 26). As a negative control, pBSK+ (vector in all of the above mentioned plasmids) was also included. A positive control reaction utilizing the pBESTluc DNA provided by the manufacturer was also included. After the reaction was completed, protein samples were run on a 12% SDS-PAGE gel, the gel was fluorographed, dried and exposed to X-ray films for appropriate time at -80°C. Table XIV shows the plasmids used, the different ORFs present in each, the expected sizes of proteins, and the observed sizes of proteins made using the S-30 system. While the predicted product of ORF4 was a 52 kDa protein, with this system a 57.5 kDa protein was made (Figure 27). Deletions spanning ORF4 made protein products almost to the expected size. pLG151 which had the entire ORF4 intact did not make a corresponding protein. In all of the plasmids used, a 20 kDa protein corresponding to the product size of ORF4.1 was present. The rest of the protein products observed did not correspond to the expected sizes of the products of ORFs 9, 10, 11, or 12.

STP protein TNT

STP protein system uses a T7 RNA polymerase promoter to direct synthesis of mRNA and a rabbit reticulocyte lysate for *in vitro* translation. The protocol used for this experiment is described in the methods section and follows the manufacturers recommendations. The plasmids used were pSP108 and pDR22. As a negative control SK+ was also included. The reactions were set up as described earlier. Three different

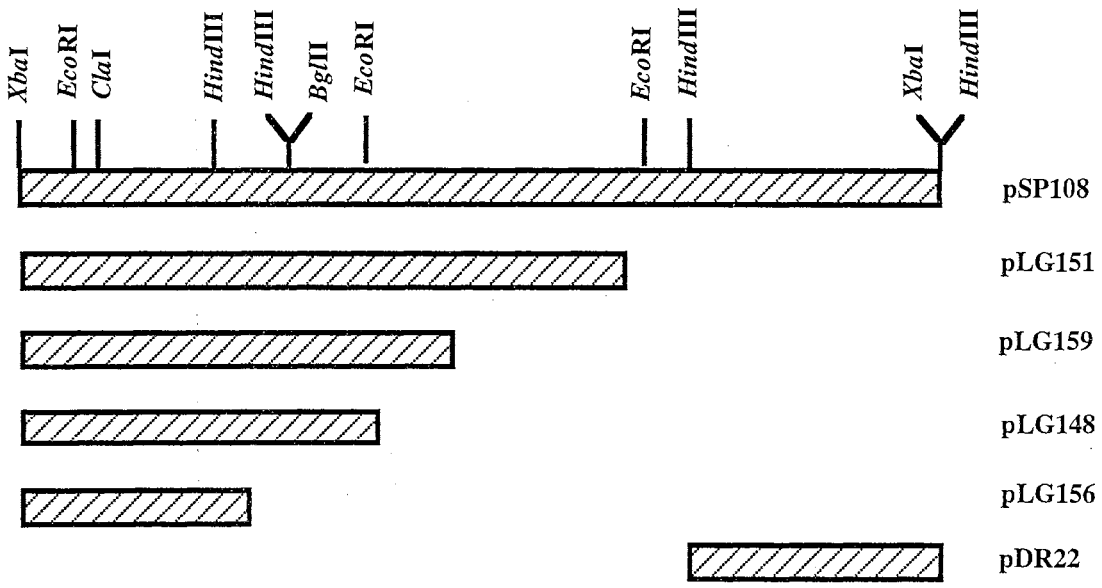


Figure 26. pSP108 and it's deletion derivatives. Boxed fragments represents insert DNA (3.25 kb or it's deletions)

TABLE XIV

in vitro TRANSCRIPTION AND TRANSLATION OF pSP108 AND IT'S DERIVATIVES USING THE *E.coli* S30 EXTRACT FROM PROMEGA

PLASMID	ORF'S PRESENT	EXPECTED SIZES (kDa)	OBSERVED SIZES (kDa)
pSP108	4, 4.1, 9, 10, 11, 12	52, 20, 14.5, 11.7, 11.9	57.5, 34.5, 20, 4.5
pDR22	11, 12	11.7, 11.9	No unique Proteins
pLG151	4, 4.1, 9	52, 20, 14.5	34.5, 20, 4.5
pLG159	4, 4.1	52, 20	57.5, 34.5, 20, 4.5
pLG148	4 (missing 94 AA), 4.1	42, 20	43, 34.5, 20, 4.5
pLG156	4 (missing 302 AA), 4.1	19, 20	27.5, 20

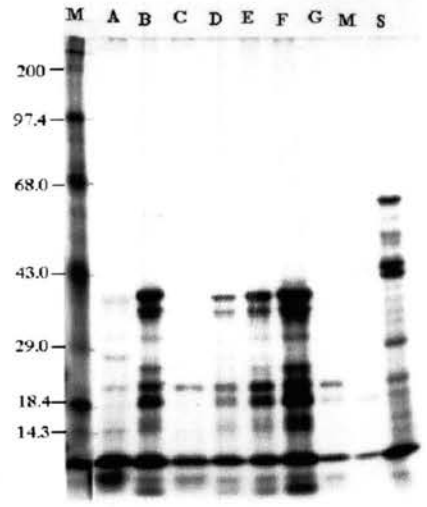


Figure 27. ***In vitro*** **Transcription and Translation using S30 Extract.** The picture shows an autoradiogram of the *in vitro* Transcription and Translation products obtained with plasmids containing the 3.25 kb *Xba*I or its deletion derivatives. The proteins were labeled with ^{35}S L-methionine and were separated on a 12% SDS-PAGE separating gel and subjected to fluorography. M. Molecular weights, given in kilodaltons; A. SK+; B. pSP108; C. pDR22; D. pLG151; E. pLG159; F. pLG148; G. pLG156; S. S30 control.

sets of reactions were set up, each using a single or combination of RNA polymerase(s). The first set utilized the T7 RNA polymerase, the second set utilized T7 and T3 RNA polymerase combination, and the third set the T3 RNA polymerase only. The reason for using the different polymerases was to show that the proteins made were directed by the T7 RNA polymerase and not by the other. After the reactions were completed, the samples were prepared for SDS-PAGE analysis and electrophoresis carried out on a 12% gel. This was followed by fluorography, gel drying, and exposure to X-ray film at -80°C. Table XV gives the expected and observed sizes of the proteins from each of the plasmids used in the different reaction types. The expected size for the ORF4 gene was 52, but the observed size was 65 kDa (Figure 28). The same was true for ORF4.1. The expected size was 20 kDa, but a protein of 26 kDa was made. pDR22 was expected to make two proteins about 12 kDA, but from this expression system, an 18 kDa protein was made. The difference in the observed protein sizes could be because of the fact that we used a eukaryotic system for translation (rabbit reticulocyte lysate). This could possibly identify other start sites and direct expression accordingly. The rest of the ORFs could not be concluded to make a protein product based on these results. Also, protein products were made only with T7 RNA polymerase and not with T3 RNA polymerase.

In vivo Identification of the Gene Products

The recombinant plasmid, pSP108, carries the 3.25 kb *Xba*I fragment that was sequenced. The T7 promoter in the vector portion (pBC SK+) of pSP108 preceding the proposed transcription site of ORF12 in pSP108 facilitated induction of transcription by T7 RNA polymerase following infection of the respective clone with M13mGP1-2 phage (79). SDS-PAGE of ^{35}S-methionine and ^{3}H-leucine labeled polypeptides synthesized in *E. coli* cells carrying pSP108 and its deletion derivatives led to the unambiguous identification of two of the six proposed products of this transfer-related region (Figure 29).

TABLE XV

in vitro TRANSCRIPTION AND TRANSLATION OF pSP108 AND pDR22 USING THE SINGLE TUBE PROTEIN SYSTEM 2 OF NOVAGEN

EXTRACT ADDED	PLASMID	ORF'S PRESENT	EXPECTED SIZES (kDa)	OBSERVED SIZES (kDa)
T7	pSP108	4, 4.1, 9, 10, 11, 12	52, 20, 14.5, 11.7, 11.9	65, 44.5, 31, 26
T7 &T3	pSP108	4, 4.1, 9, 10, 11, 12	52, 20, 14.5, 11.7, 11.9	65, 44.5, 31, 26.5, 24
T7 &T3	pDR22	11, 12	11.7, 11.9	23, 18
T3	pSP108	4, 4.1, 9, 10, 11, 12	None	No Unique Proteins
T3	pDR22	11, 12	None	No Unique Proteins

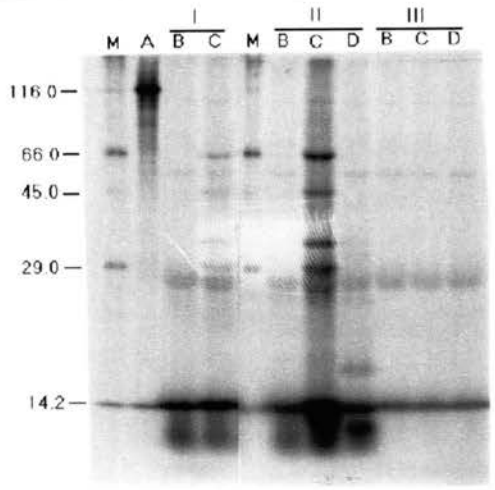


Figure 28. ***In vitro*** **Transcription and Translation using Rabbit reticulocyte lysate.** The picture shows an autoradiogram of the *in vitro* Transcription and Translation products obtained with plasmids containing the 3.25 kb *Xba*I or its deletion derivatives. The proteins were labeled with 35S L-methionine and were separated on a 12% SDS-PAGE separating gel and subjected to fluorography. Panel I represents DNA samples that were treated with the rabbit reticulocyte lysate and T7 polymerase; II. DNA samples treated with the lysate and T7 and T3 polymerases; III. DNA samples treated with the lysate and T3 polymerase. Lane A is the STP control; B. SK+; C. pSP108; D. pDR22; M. Molecular weights, given in kilodaltons.

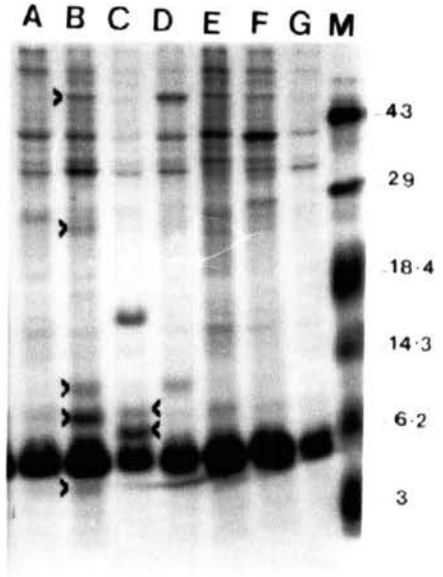


Figure 29. Autoradiogram showing SDS-PAGE profiles of protein products encoded by the 3.25 kb *Xba*I DNA segment. The 3.25 kb *Xba*I DNA fragment was subcloned into pBluescript plasmid vector, SK+, to create the recombinant plasmid, pSP108. Deletion derivatives of pSP108 were created following exonuclease III and S1 treatments. A, pBS-SK+; B, pSP108; C, pDR22 carrying a 1.1 kb *Hin*dIII DNA fragment forming the right end of the sequence; D, pLG151; E, pLG159; F, pLG148; G, pLG156. The deletion derivative plasmids, pLG151, pLG159, pLG148, and pLG156, carry approximately 2.4, 1.7, 1.4, and 0.9 kb DNA respectively from the left end of the sequence. The proteins labeled with (^{35}S)L-methionine and (^{3}H)leucine were separated on a 17% SDS- PAGE separating gel and subjected to fluorography. The molecular weights of the standards (lane M) are given in kilodaltons at right. Polypeptides presumed to be directed by the passenger DNAs are indicated by arrows.

Five polypeptides with calculated molecular masses of 52, 22, 8.2, 6.5, and 3.3 kDa appeared to be unique to cells containing pSP108 (lane B) as compared to those carrying the vector plasmid alone (lane A). Of these, the 52 and 22 kDa bands were in reasonable agreement with the sizes of expected products of ORF4 and ORF4.1, respectively. These two polypeptides were not observed in cells carrying either pDR22 or pLG156 as all or most of the DNA encoding ORF4 was not present in these plasmids. No bands corresponding to the predicted products of the other ORFs were seen in cells carrying either pSP108 or other derivative plasmids. On the other hand, the plasmid pDR22, carrying a 1.1 kb *Hind*III fragment containing the ORFs 11 and 12, seemed to encode 6.5 and 5.1 kDa products (lane C). Whether the apparent difference in molecular weights between the expected and observed polypeptides was due to post-translational processing or proteolytic degradation is not clear (75).

Purification of the ORF-4 Protein Product

In an effort to directly demonstrate a DNA relaxation property associated with the predicted product of ORF4, the gene was amplified by PCR and cloned in-frame into the protein expression vector, pMAL-p2. PCR was carried out as described earlier using as primer pairs the oligonucleotides 5'-ATGGTCATCACTAAACACTT-3' and 5'-GTAAGAACTAGTTTCTCTGAG-3'with pSP108 (3.25 kb *Xba*I) as the template. The 1.4 kb PCR product was used in ligation reaction with pMAL-p2 (*Xmn*I) as the vector, and transformed into *E. coli* ER2508 competent cells . Amp^r colonies were tested for plasmid content and following insert confirmation by restriction analysis, the recombinant plasmid was designated pVJ555. The integrity of the amplified segment was determined by DNA sequencing of both strands. pVJ555 was then used for protein purification from a large culture.

Purification of the relaxase protein from a large culture was essentially as described in the methods section. Following affinity chromatography, the protein fractions were pooled and concentrated using Amicon-10 concentrator. The concentrated protein was stored at -80°C. The fusion protein was used without cleavage for further studies.

In vitro Nicking Activity of ORF4 Gene Product

The MBP-ORF4 fusion protein from *E. coli* cells induced with IPTG was purified to near homogeneity by affinity chromatography using an amylose resin bed. Factor Xa cleavage of the fusion protein was not very efficient. Therefore, we used the MBP-ORF4 fusion protein for DNA nicking assay. The fusion protein was tested for nicking activity using supercoiled plasmids containing passenger DNA segments derived from various regions within the transposon. A typical reaction contained 20 mM Tris-HCl, pH 7.4, 100 mM NaCl, 10 mM $MgCl_2$, 1 mM EDTA, 1 mM DTT, 1 mM $CaCl_2$, 0.04 pmol supercoiled plasmid DNA, and 2 μg of MBP-ORF4 fusion protein in a 20 μl reaction volume. After overnight incubation at 37°C, reactions were stopped by adjusting the mixture to 1% SDS and 100 μg/ml Proteinase K and further incubated for 30 minutes at 37°C. The reaction mixtures were electrophoresed on a 0.7% agarose gel in 1/2X TBE and stained with 1 μg/ml ethidium bromide (Figure 30). Among the plasmids used as substrates, two were found to be nicked at the end of overnight incubation. The passenger DNAs in these pBluescript-based plasmids, pDR6 carrying a 4.6 kb *Eco*RI fragment (coordinates 5.8-10.4) and pSP108 carrying a 3.25 kb *Xba*I fragment (coordinates 4.6-8.0), contained an overlap of about 2.2 kb. This segment of DNA between coordinates 5.8 and 8.0 as defined earlier (85) and is flanked by the *Eco*RI site that is closest to the predicted start codon of ORF4 and the *Hind*III site past ORF12 in the left terminal region of Tn*5252* (Figure 3).

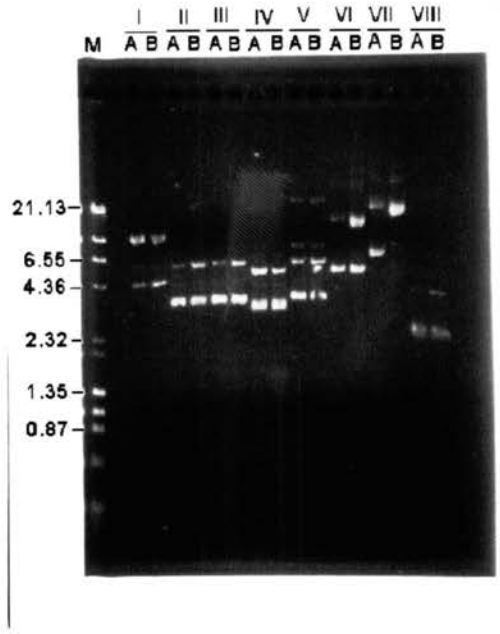


Figure 30. Localization of the nick site in Tn*5252*. Analysis of plasmids containing different segments of the transposon DNA was done to localize the site of nicking by the Relaxase fusion protein using 0.7% agarose gel. Lane M is the 1 kb ladder molecular weight standard and the molecular weights are given in kilobases at the left. Panels I-VIII represent plasmids carrying different segments of the transposon DNA. In each panel, A represents untreated DNA and B represents the same DNA sample treated with the Relaxase fusion protein. I. pVJ407; II. pSJ114; III. pVJ428; IV. pSP110; V. pSP111; VI. pSP108; VII. pDR6 ; VIII. SK+.

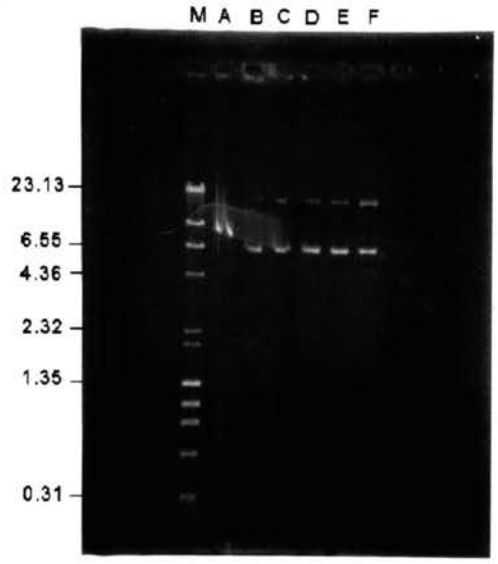


Figure 31. Effect of DNA relaxase fusion protein concentration on the nicking of pDR6. M, λ-*Hin*dIII/ϕx174-*Hae*III molecular weight markers. All other lanes contain the plasmid, pDR6. A, linearized with *Kpn*I; B, C, D, E, and F were with 0, 200, 400, 800, and 2700 ng of fusion protein, respectively.

Concentration Dependence of DNA Relaxation Activity

We sought to determine whether the activity of the relaxase protein was dependent on the concentration of the protein present. As shown in Figure 31, the DNA relaxation activity was dependent on the concentration of the protein added. However, even with the highest concentration of DNA relaxase, complete conversion of supercoiled plasmid to a relaxed form was not observed (75).

DISCUSSION

Relying on several lines of experimental evidence, the current models for the mechanism of conjugation in Gram-negative bacteria include rolling circle replication and transfer of a specific ssDNA intermediate from the donor to the recipient. An essential enzyme involved in this process is a DNA relaxase carrying type I topoisomerase activity (86). Here we describe the structural features of the predicted protein product of ORF4 of Tn*5252* that strongly resembles this group of proteins. Consistent with this expectation, the MBP-ORF4 fusion protein was found to relax only those supercoiled plasmids carrying a specific region of the transposon. Based on these results, the possible origin of transfer of Tn*5252* is localized to within a 2.2 kb DNA between the coordinates 5.8 and 8.0, flanked by *Eco*RI and *Hind*III sites, respectively. Determination of the precise target site of the DNA relaxase within region awaits further investigation.

In conjugative plasmids, transfer is thought to start with the generation of a specific DNA strand destined to be transferred from the donor to a recipient cell (86). The DNA relaxase is presumed to initiate this process by catalyzing strand- and site-specific cleaving-joining of DNA at the origin of transfer. Of the three conserved domains in DNA relaxases, a tyrosine residue in motif I is presumed to take part in the covalent attachment of the protein to the 5'-phosphate at the nick site while a histidine residue in motif III forms a part of the catalytic center (53). A serine in motif II seems to be responsible for specific

recognition of the target region for nicking. Interestingly, the conserved sequence motifs seen in DNA relaxases also have been shown to be present in the replication initiator proteins playing crucial roles in the rolling circle mode of replication of small isometric ssDNA phages, several mobilizable ssDNA plasmids found primarily in gram-positive bacteria, and plant gemini viruses (34). Based on these observations, we speculate that the conjugal process of Tn*5252* may also involve transfer of a specific ssDNA strand.

Little is known at present with regard to the mechanism of intercellular movement of conjugative transposons. In Tn*916*, ORF23 has been reported to carry local homology to the relaxase protein of the plasmid, ColE1 (24). Also, results from another laboratory (64) seem to reinforce the notion that a specific ssDNA of Tn*916* is transferred during conjugation. These results which parallel our studies seem to indicate that the overall mechanics of the actual process of conjugation of all classes of streptococcal conjugative transposons may be similar.

Two major transcriptional units, based on the presence of potential initiation signals, seem to be present in the sequence given in this region. There was no obvious indication that these two are controlled by a common regulator. On the other hand, the presence of one or more products of the transcriptional unit comprising of ORF10, ORF9, ORF4, and ORF4.1 seems to reduce or abolish the expression of the other transcriptional unit of ORF12 and ORF11. Further studies are needed to understand the functional regulation of this interesting element.

CHAPTER VI

CONCLUSIONS

The major focus of this work was to identify some of the transfer related genes from the left terminus of Tn*5252* and assign functional roles to them. As an initial step, sequence data was obtained of a region in the left terminus shown to be involved in the conjugal transfer of Tn*5252* (38). Database searches led to the identification of several ORFs, of which two have been studied in detail in this work. The predicted product of ORF3 of Tn*5252* was found to be significantly similar to a variety of prokaryotic repressors and regulators. Upon introducing a mutation in ORF3 by a heterologous insertion, the resulting mutant was found to be deficient in conjugal transfer. The protein product of ORF3 bound to sites upstream of ORFs 1, 3, 4, and 10 which probably code for integrase, regulator, DNA relaxase and an as yet unidentified protein, respectively.

Another focus of this work involved the predicted product of ORF4 which strongly resembled DNA relaxases of both gram-negative and gram-positive systems. It has been shown earlier that a mutation in ORF4 led to the loss of conjugal transfer properties (38). The purified protein of ORF4 was found to relax supercoiled plasmids carrying a specific region of the transposon. This region could possibly contain the origin of transfer, *oriT*. The precise target sequence of the *oriT* of Tn*5252* is yet to be identified.

Very little is known regarding the mechanism of conjugal gene transfer in gram-positive bacteria. In the absence of any known model for the transfer of DNA (plasmid or transposon) in a gram-positive system, I am using a model proposed by Wilkins and Lanka (86) for the transfer of gram-negative plasmids such as IncI1 and IncP plasmids to speculate on the conjugal transfer mechanism of Tn*5252* from a donor to a recipient cell based on the information accumulated from this study.

The general model of bacterial conjugation proposes that a specific plasmid strand is transferred from the donor cell to the recipient cell in the 5' to 3' orientation, retaining the complementary strand in the donor. This process is initiated by a single strand nick at *oriT* which is catalyzed by the DNA relaxase bound covalently to the unique 5' terminus at the nicked *oriT* site, followed by rolling circle replication and transfer of a single stranded DNA intermediate from the donor to the recipient. Synthesis of the replacement strand in the donor and complementary strand synthesis in the recipient cell completes the process.

In this study we have identified a regulatory region of Tn*5252* (74). The arrangement of the other genes in the left terminus (38) also suggests that a similar mechanism is possible with Tn*5252*. Convincing evidence comes from the fact that a DNA-relaxase has been identified and shown to nick a specific sequence in the left end of this element (75). Also, in this study we have shown that the ORF3 protein product binds upstream of several ORFs in the left terminus including the relaxase gene. This indicates that the ORF3 protein may possibly be the master regulator of genes involved in the DNA metabolism during the conjugal transfer of Tn*5252*.

The information obtained in this investigation should provide a framework for approaching several other interesting questions regarding the intercellular transmission of Tn5252 such as (a) the nature of the inducer, (b) early events occurring during conjugation, and (c) the nature of the DNA-relaxase protein interaction.

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