STRUCTURAL AND FUNCTIONAL ANALYSIS OF A REGION CONFERRING RESISTANCE TO UV LIGHT IN THE STREPTOCOCCAL CONJUGATIVE TRANSPOSON Tn*5252*

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Tn*5252*

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CHAPTER I

INTRODUCTION

In recent years, the worldwide appearance of multiple drug resistance in clinical isolates of *Streptococcus pneumoniae*, the primary etiological agent of pneumonia and the most frequent cause of otitis media and bacteremia in children (8), has been of special concern. Some strains were found to be resistant to more than eight drugs (13, 16). Efforts by several groups to isolate endogenous R-plasmids, the most common vehicles of antibiotic resistance genes, from these strains were unsuccessful (10, 66, 83, 102). Later studies showed that the antibiotic resistance determinants of *S. pneumoniae* were associated with a novel class of genomically inserted elements called conjugative transposons (16, 24).

Conjugative transposons are mobile elements capable of intercellular transfer by a DNase-resistant process requiring cell-to-cell contact. These elements provide an efficient method of horizontal gene transfer and are considered to be chiefly responsible for the dissemination of multiple antibiotic resistance in clinical streptococci (73). The initial focus of our laboratory was on the study of one of such elements, Tn*5253.*

Tn*5253*, formerly called the Ω(cat tet) element, is a 65.5-kb DNA fragment originally identified as a chromosomal insertion in the plasmid-free clinical isolate of *S. pneumoniae* BM6001 (77). This element was found to be self-transmissible and encode resistances to chloramphenicol and tetracycline. Tn*5253* has also been shown to insert at a specific target site in the pneumococcal genome (93). Using directed insertional mutagenesis, a series of recombinant plasmids carrying DNA from Tn*5253* have been constructed in *E. coli*. Physical analysis of the passenger DNA from these clones resulted in a detailed restriction map of the element that made possible the localization of drug resistance determinants and the identification of its junction and target regions in the pneumococcal chromosome (92-94).

Later studies in this laboratory demonstrated that Tn*5253* is a composite structure of two independent conjugative tranposons, Tn*5251* and Tn*5252* (5). Tn*5251* is an 18-kb element that codes for tetracycline resistance. Interestingly, Tn*5251* was capable of independent conjugative transposition when removed from the parental element. This small element was also able to insert at many sites of the pneumococcal chromosome and showed structural and functional similarities to the well-studied group of Tn*916*-like elements (5). On the other hand, deletion of this element from the middle region of Tn*5253* did not affect the intra- and interspecific transfer of the remaining 47.5-kb fragment called Tn*5252* (5). Tn*5252* carries the chloramphenicol resistance determinant. Functional similarities between Tn*5252* and the parental element Tn*5253*, such

as target specificity, suggested that Tn*5252* might have served as a receptacle for the insertion of the smaller element Tn*5251* at a later time giving rise to the larger composite transposon Tn*5253*.

Since Tn*5252* exhibits unique physical and functional features that differ from the ones of the well-studied Tn*916*-like elements, our group sought to study the genetic and molecular organization of this element and understand the nature and evolution of this class of chromosome-borne elements.

A functional map of Tn*5252* was obtained by insertion mutagenesis using a heterologous plasmid. Using this strategy, several transfer-related regions were identified (39, 40). The transfer-related genes seemed to be clustered towards the termini of the element. Based on these results, extensive DNA sequence information from the terminal regions of the element was obtained. Sequence from about 6 kb of the left end revealed the presence of 13 open reading frames (ORFs). Proteins of two of these ORFs have been purified and their activity demonstrated. One of these ORFs encodes a regulatory protein and the other one encodes a DNA relaxase (86, 87). Sequence of about 16 kb from the right end of the transposon revealed the presence of DNA transport genes in an operon-like structure and a DNA cytosine-methyltransferase gene. The product of the latter gene was shown to be functional (2, 70).

On the other hand, several regions around the central portion of the transposon were found to be non-essential for conjugative transposition. It is thought that some of the genes located in these regions code for factors that

may contribute in vivo to the virulence of strains carrying the transposon.

Recent sequence data of a region within a 4.55-kb *Eco*RI fragment located on the left region of Tn*5252*, immediately adjacent to the 6 kb DNA at the left terminus whose sequence has been obtained, revealed the presence of two ORFs displaying significant similarities to *umuC* and *umuD* homologues. These genes are involved in protection against ultraviolet irradiation (UV) and induced mutagenesis. Usually, these genes are part of dicistronic operons (*umuDC*) that encode the structural genes for error-prone repair during the SOS response (80).

The finding of such genes on Tn*5252* is interesting since it has been proposed that *S. pneumoniae* is naturally devoid of UmuDC-like functions (55). Therefore, the presence of these functions on the transposon may contribute to the protection of *S. pneumoniae* strains carrying this element. This work was initiated to test this possibility. The main objective of this study was to investigate the role of this region in UV damage protection and induced mutagenesis response in *S. pneumoniae*. Also, an *in vivo* mutation was created to study the role of these ORFs in the conjugal transposition of Tn*5252*. Finally, the DNA sequence of the 4.55-kb *Eco*RI fragment was completed in order to obtain the structural organization of this region.

CHAPTER II

LITERATURE REVIEW

Streptococcus pneumoniae

S. pneumoniae, also called pneumococcus, is one of the most studied microorganisms ever since it was first isolated by Pasteur in 1881 (20). In the early 1900s, it was used in the famous experiments of Griffith and Avery that lead to the important discovery that DNA was the carrier of genetic information (4). Some of other important discoveries resulting from the study of this microorganism include the role of capsule in avoidance of phagocytosis, the therapeutic effect of penicillin, the induction of antibodies by polysaccharides (20), and the mismatch repair system (31). Even though there have been an incredible number of studies on pneumococcus, many questions about the mechanisms that lead to the high morbidity and mortality caused by this microorganism are still unanswered.

Pneumococci are gram positive cocci that occur mainly in pairs or small chains. The cells show a typical lancet shape. Only strains that are encapsulated are virulent. Interestingly, after repeated subculturing in the laboratory, the

capsule can be lost (8). Pneumococci are facultative anaerobes that are fastidious in their cultural requirements. The addition of blood to the medium supplies the enzyme catalase that is absent in pneumoccoci (90). On blood agar, pneumococcal colonies exhibit alpha hemolysis.

About 5% to 50% of the population carry *S. pneumoniae* in the upper respiratory tract. Pneumococcal diseases are more often the result of endogenous infection through lowered host resistance than the result of exogenous transmission. The incidence of pneumococcal diseases is higher in people over 60 years old and in infants under 2 years of age. *S. pneumoniae* causes 60% to 70% of all bacterial pneumonia, especially community-acquired pneumonia. In addition, it is the most common cause of otitis media in infants and children. *S. pneumoniae* is the most frequent cause of bacterial meningitis in adults, especially in immuno-compromised and debilitated patients, and the second most common, after *Hemophilus influenzae*, in young children (20). In developing countries, a large number of children under five years die every year from pneumococcal pneumonia. In spite of the availability of a vast variety of antibiotics, the mortality due to pneumococcal bacterimia has remained stable between 25% and 29% during the last four decades (20).

Antibiotic Resistance in Pneumococcus

Traditionally, bacterial antibiotic resistance has been linked to plasmid borne genes. This type of resistance is common in all the clinical streptococcal species studied with the intriguing exception of *S. pneumoniae*. Although pneumococcus can accept and maintain a variety of plasmids from other streptococci, endogenous extrachromosomal elements have only been rarely observed in this species.

When multiple resistant strains of clinical pneumococcus started to emerge in the late 1970's (36), efforts to isolate resistance plasmids from these strains were unsuccessful (10, 66, 83, 102).

Shoemaker *et al.*, (77) provided the first convincing evidence for the chromosomal location of the resistance determinants. They demonstrated that the chloramphenicol resistance (*cat*) and tetracycline resistance (*tet*) of the BM6001 strain were due to a heterologous DNA insertion in the bacterial chromosome. They observed that transformation of wild-type *S.pneumoniae* Rx1 with DNA of the multiply-antibiotic resistance strain BM6001 gave rise to Cm^r and Tc^r colonies at frequencies that were lower of those expected for transformation of homologous point markers. Also, it was observed that *cat* transforming activity, which was relatively insensitive to shearing of donor DNA, co-sedimented with chromosomal DNA markers of both sheared and unsheared lysates (77). This large heterologous piece of DNA was called Ω*cat tet* element.

It was later renamed Tn*5253*. Elements with similar characteristics were found in other strains of *S. pneumoniae* (17, 18), in other streptococcal species such us *Streptococcus pyogenes* (47) and *Streptococcus agalactiae* (35), and in *Enterococcus faecalis* (24, 28).

Several studies revealed that these heterologous elements, including the Ω*cat tet* element , were able to transfer to recipient strains on membrane filters by a DNase resistant process resembling classical conjugation. The fact that this process was DNase resistant eliminated the possibility of transfer by transformation or generalized transduction (78). The transfer mechanism of these streptococcal elements was proven to occur both intra- and interspecific (9, 13, 17, 34, 78). These elements constituted a novel group of antibiotic resistance vehicles termed "conjugative transposons".

Conjugative Transposons

Conjugative transposons have been isolated from a variety of streptococcal species. These elements vary in size from 16 kb to over 60 kb. All of the streptococcal conjugative transposons studied so far carry a tetracycline resistance determinant (*tet*), many of them also carry other multiple drug resistances. In general, conjugative transposons transfer from one bacterial cell to another by a process requiring cell to cell contact. Their frequencies of transfer range from 10^{-4} to 10^{-9} per donor (74) being noticeably lower than the

transfer frequencies of plasmids which range from 10^{-1} to 10^{-3} per donor (84). The transfer process is *recA* independent and DNase-resistant. Transconjugants, on the other hand, behave like the parental strain being able to mobilize the acquired element.

Conjugative transposons display a set of unique characteristics that separate them into a novel group of gene transfer systems. In spite of their uniqueness, they share some features with other gene transfer systems such as transposons, phages and plasmids. Like the commonly studied transposons from *Escherichia coli,* Tn*5* and Tn*10*, conjugative transposons excise from and integrate into DNA molecules. However, the mechanisms of integration and excision are different. Conjugative transposons do not duplicate the target site when they integrate into the recipient genome (65, 73, 74)

Transposition of conjugative elements resembles integration and excision functions of temperate bacteriophages that have a circular intermediate. In fact, nucleotide sequences of genes involved in integration and excision share similarity with genes that code for enzymes of the lambda integrase family (39, 60, 88). However, in contrast to the lamboid phages, conjugative transposons do not form viral particles.

Like plasmids, conjugative transposons have a covalently closed circular intermediate in conjugation. However, unlike plasmids, these elements lack autonomous replication functions and are independent of host recombination systems (65, 74).

Classification of Conjugative Transposons

Conjugative transposons were first identified in Gram-positive bacteria (5, 13, 16, 78). In recent years, a variety of conjugative transposons has also been identified in Gram-negative bacteria.

Conjugative Trasposons of Gram Negative Bacteria

A number of conjugative transposons have been identified in several species belonging to the genera *Bacteroides, Kingella, Neisseria, Salmonella* and *Vibrio* (68, 95, 96).

Conjugative transposons of the Tn*916* family have been found in *Kingella spp* and in *Neisseria gonorrhoeae* (89). Tn*916* was originally found in *E. faecalis*, a Gram-positive microorganism. Studies have shown that this element was capable of mobilizing itself among Gram-positive and Gram-negative bacteria. Therefore, the presence of this type of elements in these microorganisms is not very surprising.

Non-replicating Bacteroidal Units. A distinct group of conjugative transposons has been found in *Bacteroides*, an anaerobic Gram-negative microorganism. This group of elements are called Non-replicating Bacteroidal units (NBUs) (68). NBUs are large elements; they range in size from 65 to over

150 kb. Most of them carry resistance genes for tetracycline. These elements are able to integrate into co-resident plasmids and transposons and mobilize them in *cis* (68).

Conjugative Trasposons of Gram Positive Bacteria

Tn*916*/Tn*1545* Type Elements. Members of this family are small elements that range in size from 18 kb to 25 kb. Transposons that belong to this family usually carry the *tet*M determinant that confers resistance to tetracycline. This type of conjugative transposons was the first discovered and, therefore, the most studied one.

Tn*916* was first found in the chromosome of the hemolytic multidrug resistant *E. faecalis* DS16 (24). Tn*916* has been shown to intracellularly transpose from the chromosome to the hemolysin plasmid, pAD1. It was also able to transpose intercellularly via conjugation to a number of sites on the recipient's chromosome (24).

Intercellular transfer of Tn*916* occurs by a *rec* independent process. Transconjugants resulting from independent conjugation experiments were found to have different transfer potentials with transfer frequencies ranging from 10^{-4} to 10^{-9}. Transconjugants were also found to differ in the number of element copies acquired (28). DNA sequencing of this element revealed the presence of short direct and inverted repeats at the termini. Excision of the element was

precise and insertion of the transposon did not generate duplications at the target sites (11). The transposition mechanism of Tn*916* involves a circular intermediate (73).

Tn*1545* was found in the chromosome of *S. pneumoniae* BM4200, a multiple antibiotic resistant strain. Besides carrying the antibiotic resistance determinant for tetracycline (*tet*), it also carried the erythromycin (*erm*AM), kanamycin (*aph*A) and chloramphenicol (*cat*) resistance determinants (17). This 25 kb element shares substantial homology with Tn*916* having similar structural and mechanistic features (60). Predicted amino acid sequence of the DNA sequence of the termini of this element has been observed to be structurally and functionally homologous to the integrase and excionase of the lamboid phages (60).

Members of this family are able to transfer to different Gram-positive and Gram-negative bacteria having a remarkably broad host-range (15).

Tn*3701*/Tn*5253* Type Complex Elements. These elements are large complex conjugative transposons that encode several antibiotic resistances. They usually carry an internal region with extensive homology to Tn*916* type of elements. Unlike the Tn*916* family of elements, the larger conjugative trasposons show target specificity.

Tn3701 was first identified in *S. pyogenes* A54. It is a 50 kb element and carries the determinants for tetracycline, chloramphenicol and erythromycin

resistance (47). The *erm-tet* determinants are located within a 20-kb fragment homologous to Tn*916* (47). This fragment was able to independently transpose from a plasmid to different sites on the chromosome but lacked conjugative properties (47). It has been proposed that the reason for the lack intercellular transfer is due to the insertion of the *erm* determinant in a gene necessary for conjugation (48).

Other members of this family are Tn*3951*, a 67 kb element found in the chromosome of *S. agalactiae* B109, that also carries determinants for tetracycline, chloramphenicol and erythromycin resistances, and Tn*5253* (35). The nature of the latter element will be discussed in more detail later in this chapter.

The Lactococcal Element. A composite transposon, unrelated to the Tn*3701*/Tn*5253* family, has been identified in *Lactococcus lactis*. This element is called Tn*5276* and carries a gene involved in sucrose metabolism and a gene encoding nisin resistance (64).

Tn*5253*

Based on linkage to chromosomal markers, Shoemaker and Guild showed that the chloramphenicol and tetracycline resistances of *S. pneumoniae* BM6001 were present in a heterologous piece of DNA in the bacterial chromosome (77). This element was initially termed Ω*cat tet* and then renamed as Tn*5253*. This is a 65.5-kb element that has been shown to site-specifically insert in the chromosome of *S.pneumoniae*. The conjugative transfer efficiency in filter mating experiments was observed to be between 10^{-6} to 10^{-7} (83).

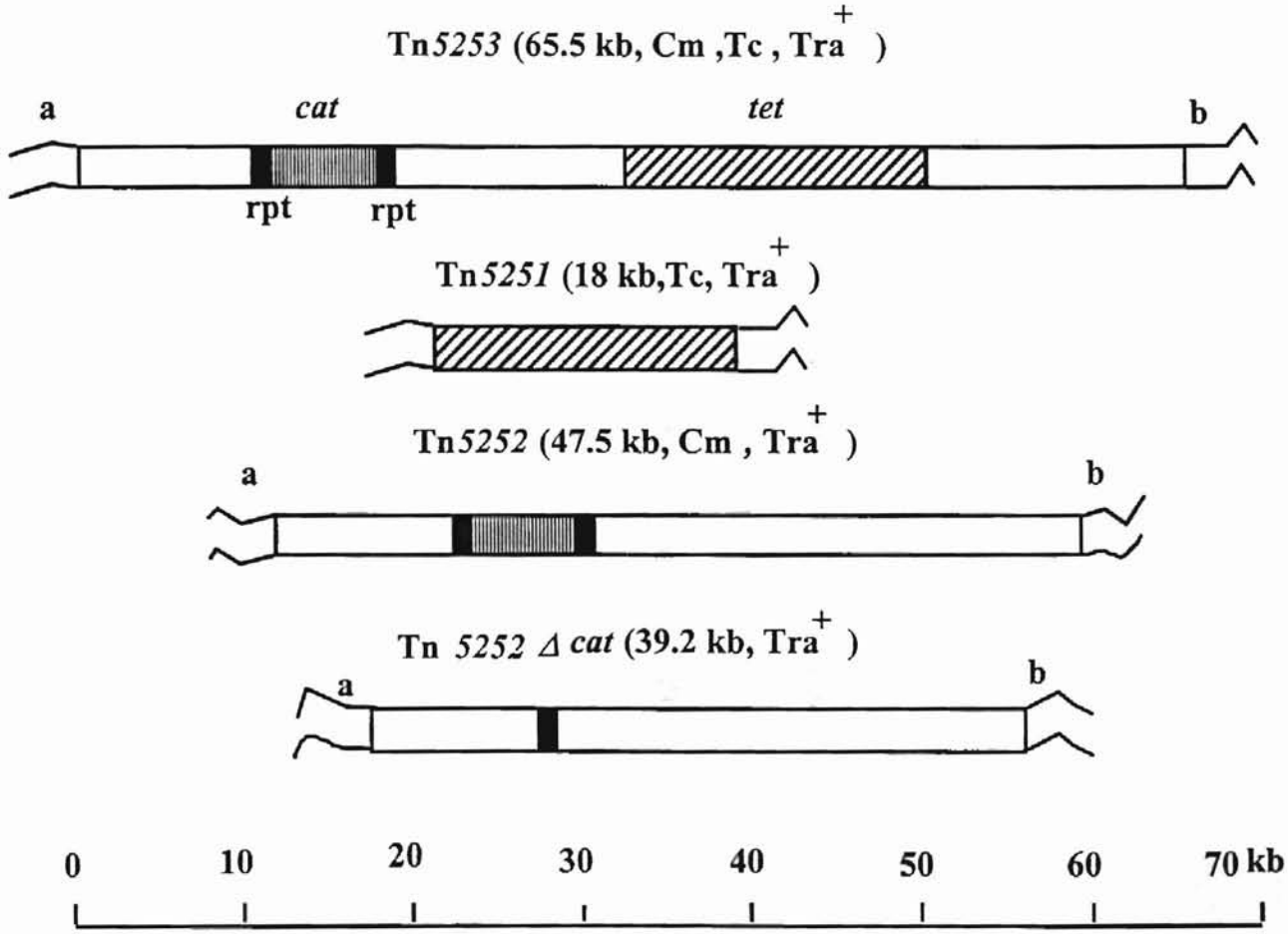
Using directed insertion mutagenessis, Vijayakumar *et al.* were able to obtain several *E. coli* plasmids carrying large fragments of DNA from Tn*5252* (93). The vector plasmid employed was pVA891 (53). This vector carries Em and Cm resistance determinants that are expressed in *E. coli*. In pneumococcus, Cm does not express but Em is expressed when the plasmid is integrated into the chromosome. Using this approach, the localization of the drug resistances was obtained. Also, the termini and the target site of the transposon in the chromosome were cloned and identified (94). Later experiments showed that Tn*5253* was a composite element formed by two independent conjugative transposons, Tn*5251* (Tc, 18 kb) inserted within Tn*5252* (Cm, 47 kb.) (5). DNA hybridization experiments and restriction mapping showed that the 18 kb DNA fragment carrying the *tet* determinant was homologous to Tn*916* (47). To prove that Tn*5251* was functionally similar to Tn*916*, Ayoubi *et al.* cloned a 23 kb *Xba*I

fragment from Tn*5253* into the pVA891 vector. When this construct was introduced into *S. pneumoniae*, it was observed that the 18 kb element transposed into different sites in the chromosome. This element was also shown to be capable of intercellular transfer. On the other hand, no significant homology was detected between the two independent transposons forming Tn*5253*. The DNA beyond Tn*5251* within Tn*5253*, now termed Tn*5252*, exhibited unique structural and functional properties that have been the focus of studies in this laboratory (Figure 1).

Tn*5252*

Unlike the Tn*916*/Tn*5251* class of elements, Tn*5252* was shown to insert at a unique site in the pneumococcal chromosome (92, 93). Therefore, it was assumed that a specific region in the pneumococal chromosome served as an *att* site for this element. To determine whether the integration of the element into the chromosome was due to homologous recombination, a strain carrying a chromosomal insertion of Tn*5252*, SP1000, was used as donor in filter-mating experiments (5). Different species of streptococci were used as recipients. DNA of the transconjugants was hybridized to probes carrying either the left or the right junction of Tn*5252*. In all the transconjugants, each probe hybridized to a single fragment of identical size, indicating that this element was target specific, even in non-pneumococcal species (92).

Figure 1. Structure of the composite transposon Tn*5253*. Removal of Tn*5251* from the larger transposon results in the formation of Tn*5252*. Spontaneous loss of *cat* occurs at a detectable frequency giving rise to Tn*5252*Δ*cat* that is still transfer-proficient.


Tn5253 (65.5 kb, Cm ,Tc , Tra+)
a
cat
tet
b
rpt
rpt
Tn5251 (18 kb,Tc, Tra+)
Tn5252 (47.5 kb, Cm , Tra+)
a
b
Tn 5252 Δ cat (39.2 kb, Tra+)
a
b
0
10
20
30
40
50
60
70 kb

DNA sequence analysis of plasmids carrying the termini and the target regions of the trasposon revealed that a 72 bp segment of the chromosome target region, *att*B, was present at both ends of the transposon (92). The copies at the ends of the trasposon, *att*L (at the left end) and *att*R (at the right end), were 73 and 74 long, respectively. Besides the small difference in size, *att*L and *att*R contained some minor sequence differences, especially in the middle. No inverted repeats were found at the termini of Tn*5252*. It was also shown that the left 72 bp repeat was part of the element while the right repeat belong to the host chromosome implying that the integration of Tn*5252* was a conservative recombination event that resembles the site-specific insertion of phages (92).

In order to identify the transfer-related regions within the transposon, in vivo insertions and deletions were created using directed insertion of a non-replicating plasmid vector as a reporter cassette. For this, the plasmid vector pVA891, was used to generate a series of clones carrying fragments from different regions of Tn*5252* (93). After transformation, these molecules were expected to insert into the chromosome of the strain SP1000, carrying the element, in a homology dependent recombination reaction (61). Using this approach, several insertion and deletion mutants were generated. In order to detect transfer-deficient (Tra^-) mutants, pneumococcal cells carrying the mutations within Tn*5252* were used in filter mating experiments with *S. pyogenes* as recipients. Most of the Tra^- mutants carried mutations in the terminal regions of the element whereas mutations in the middle of Tn*5252* were

found not to affect the transfer (39). These results were consistent with the findings that Tn*5251* and the *cat* gene flanked by IS elements were located in the middle region of the transposon suggesting that smaller elements like Tn*5251* were added later to larger ones like Tn*5252* to generate composite larger elements such as Tn*5253.*

In order to characterize the transfer-related regions, extensive DNA sequence information from the terminal regions of the element was obtained. Following subcloning of a variety of restriction fragments from these regions, DNA sequence of about 6 kb of the left end and about 14 kb from the right end was obtained (1, 2, 70, 87). Computer analysis of the sequence of the left end of the transposon revealed the presence of about twelve possible ORFs, including a site-specific recombinase or integrase. Further analysis of this region led to the purification of two proteins, a DNA relaxase and a regulatory protein (86, 87). The DNA relaxase has been shown to nick in a site-specific manner within a region of the trasposon (86). The regulatory protein has been observed to bind to sequences upstream of the integrase gene, the DNA relaxase gene, and to its own promoter (87).

Nucleotide sequence from the right end of the transposon revealed the presence of an operon-like structure consisting of a least ten genes (1). The predicted proteins of these genes showed significant similarities to DNA transport proteins. One of these is predicted to form transmembrane domains typical of proteins involved in transferring DNA across the membrane (1). Also, on the

right-most end of the transposon the presence of a functional methyltrasferase has been demonstrated (70).

In order to elucidate the function of the region located at the right end of the DNA relaxase gene, sequence analysis was performed. Preliminary studies revealed the presence of a determinant involved in UV light resistance and SOS mutagenesis.

SOS Response and Mutagenesis in Prokaryotes

DNA-damaging agents ranging from UV light to reactive oxygen species constitute a constant threat to an organism's genome. Therefore, organisms have evolved a variety of repair mechanisms. The repair systems are likely to be as complex as the replication apparatus itself, which indicates their importance for the survival of the cell (49).

Repair systems often can recognize a range of distortions in DNA as signals for action. In addition, an organism may have several systems that are able to deal with DNA damage. In bacteria, the most common mechanisms are DNA repair by reversal of damage or direct repair, excision repair, mismatch repair, and recombination repair, also known as retrieval system (25). When DNA damage cannot be repaired in an error-free manner, a mutagenic event may occur. Bacteria have evolved an inducible coordinate system, termed the SOS system, in response to damages that challenge the integrity of the genome (25).

Direct repair

This mechanism involves the direct reversal of the damage. A common example is photoreactivation of pyrimidine dimers in which the enzyme photolyase, encoded in *E. coli* by the *phr* gene, is able to remove the offending covalent bonds (67).

The reversal of damage in DNA is the most direct mode of DNA repair. In most of the cases only a single gene product is required and the process occurs more rapidly than in multistep biochemical pathways such as excision repair (25).

Excision Repair

There are multiple excision repairs systems in a single cell; they vary in their specificity and in the heterogeneity of the segments of repaired DNA. Usually, they handle most of the damage in the cell. Based on the length of the repaired DNA they are classified into very short patch repair (VSP), short repair, and long-patch repair. The latter two excision repair systems involve the *uvr* genes (25).

The *uvr* system of excision repair in bacteria includes three genes: *uvrA, uvrB* and *uvrC* that code for components of a repair endonuclease. First, the UvrAB complex recognizes pyrimidine dimers or other bulky lesions. Then, UvrA

dissociates, and UvrC binds UvrB. The UvrBC combination makes an incision on each side of the damage. UvrD is an helicase that helps unwind DNA to allow release of the single strand between the two cuts. The average length of the excised DNA is 12 bases (71).

Mismatch Repair

Mismatched base pairs in DNA can arise mostly due to replication errors. During mismatch repair, the correct base of the mispair is located in the parental strand of the newly replicated DNA. Proper correction of the mismatch contributes to the maintenance of the fidelity of the genetic information (25).

The best understood bacterial mismatch repair systems are the methyl-directed mismatch repair system of *E. coli* (30) and the related Hex-dependent mismatch repair system of *S. pneumoniae* (12, 45). The excision tracts associated with these pathways can be 10^3 bases or more, although shorter patches can also be repaired using this system.

The first evidence for the repair of mismatched bases in prokaryotes came from studies in transformation of *S. pneumoniae* (12). During transformation of pneumococcus, the double stranded DNA from the donor is converted to single stranded fragments (45). By a recombinational process, the donor DNA replaces homologous segments in the recipient's chromosome to generate a heteroduplex region that may contain a mismatch (23). Mismatch repair occurs preferentially

on the donor strand and accounts for the differences in transformation frequency in *S. pneumoniae* (21). The higher the repair efficiency, the lower the transformation efficiency.

S. pneumoniae mutants that showed the same transformation frequency for high- and low-efficiency markers were designated *hex* mutants for heteroduplex repair deficiency. Interestingly, *hex* mutants were found to have an elevated spontaneous mutation rate (45), raising the possibility that hex-dependent mismatch-repair system may play a role in mutation avoidance (45).

Retrieval Systems

Retrieval systems have been also termed recombination repair because their activities overlap with those involved in genetic recombination. When damage remains in a daughter molecule, and replication has been forced to bypass the site, a retrieval system uses recombination to obtain another copy of the sequence from an undamaged source (49).

In *E. coli* deficient in excision repair, mutations in the *recA* gene essentially abolish the remaining repair including the rertrieval systems. Attempts to replicate DNA in *uvr-recA*- cells only produced fragments of DNA with sizes that correspond with the expected distance between thymine dimers. Therefore, RecA function is very important for the proper activity of the retrieval systems (80).

SOS Response

The SOS response consists in a regulon of over 20 unlinked genes that are involved in DNA-damage tolerance and in repair. This system is induced after the cells are exposed to DNA-damaging agents and triggers a complex series of phenotypic changes. One component of this complex response is an error-prone mechanism called SOS mutagenesis (25). The SOS mutagenesis system is designed to allow replication to continue through lesions that otherwise could result in lethal interruption of DNA replication due to the inability of the DNA polymerase to use damaged DNA as template. In exchange for increased survival, the cell pays a cost of an elevated mutation rate. The mechanism that allows the DNA polymerase to insert nucleotides opposite a misinstructional or noninstructional lesion is termed translesion DNA synthesis (80).

E. coli has been used as a model system to study the molecular basis of UV and chemical mutagenesis. Studies on this microorganism have offered detailed insights into the proteins involved in this process and into their mechanistic roles.

Regulation of the SOS Response

In *E. coli*, the *recA*$^{+}$ and the *lexA*$^{+}$ gene products are needed for regulation of the SOS response (80).

LexA is a small protein (22 kD) that is relatively stable in untreated cells. It functions as a repressor for many operons. The target genes for LexA repression include many repair functions such us *uvrA, uvrB, uvrD, umuCD* and the *din* genes (25, 80). LexA also represses *recA* and itself (50).

LexA represses its target genes by binding to a 20 bp stretch of DNA called SOS box. Binding of LexA in the promoters of the SOS response genes interferes with the binding of RNA polymerase (51). Not all the SOS boxes are identical; they have a consensus sequence with 8 conserved positions (25). SOS boxes overlap with the promoters of the controlled genes. At the *lexA* locus, there are two adjacent SOS boxes (25).

Besides important role in homologous recombination and recombination repair, RecA plays an essential role in regulation of the SOS response. RecA is able of forming a helical, multimeric nucleoprotein filament around single stranded DNA (ssDNA) that is central to its different functions in the cell (80).

RecA is activated following DNA damage. The inducing damage can be produced by exposure to UV (the most studied one) or can be caused by crosslinking or alkylating agents. In addition, deprivation of thymine, addition of drugs that stop replication, or mutations in *dna* genes can activate RecA (19). Activation of RecA implies the formation of the nucleoprotein filament by binding to ssDNA generated by the cell's attempts to replicate damaged DNA (19, 72). The activated form of RecA, denoted as RecA*, functions as a coprotease that mediates the cleavage of LexA. The cleavage reaction is unusual in the sense

that LexA has a latent protease activity that is activated by RecA*. After activation, LexA undergoes autocatalytic cleavage inactivating its repressor function. This derepression effect coordinately induces all the operons to which LexA was bound, resulting in the induction of the SOS regulon (25).

It is thought that when the cells recover from DNA damage, the amount of inducing signal decreases causing RecA* to revert to RecA. This results in accumulation of uncleaved LexA, repressing again the different genes involved in the SOS response. Differential affinity of the LexA for the promoters of the regulon provides further modulation. This allows some genes to be fully active at lower levels of DNA damage than others (25).

SOS-Dependent Mutagenesis

The SOS mutagenesis process requires the expression of *umuD* and *umuC* genes. These genes usually form dicistronic operons. *E. coli* strains carrying *umuD* or *umuC* mutations were found to be largely nonmutable by a variety of agents including UV irradiation (6, 97). It was also observed that these mutants showed increased sensitivity to killing by DNA-damaging agents such us UV light (6, 97). Although these mutations increased sensitivity to UV radiation, the mutants were much less sensitive than *uvr, recA* or *lexA* mutants. *umuD* and *umuC* mutants differ from *recA* and *lexA* mutants in that they are still able to

express a variety of SOS responses such as induction of λ prophage, increased synthesis of RecA protein, and filamentous growth (25)

umuD and umuC are organized in an operon and encode products of about 15 kb and 45 kb, respectively (80). UmuD protein is present at about 180 copies per cell in uninduced *lexA*$^+$ strain but is present at about 2400 copies per cell in lexA- strain. The levels of UmuC protein in a *lexA*$^-$ strain are about 12-fold lower than UmuD protein (80).

Considerably homology has been found between UmuD and the COOH-terminal domains of the LexA repressor and the repressors of phage λ, 434, and P22 (80). Later experiments demonstrated that UmuD is cleaved in a RecA-dependent fashion after cells have been exposed to a SOS-inducing treatment. The finding that the coprotease activity of RecA* is able to mediate the autocleavage of UmuD to a smaller form called UmuD′ came from immunoblotting experiments using antibodies against UmuD proteins. After UV irradiation, the smaller form of UmuD was detected in the immunoblotts (80). Autodigestion of UmuD was shown to remove the first 24 amino acids of the protein (76). It is thought that UmuD and LexA interact with the RecA nucleoprotein filament in a similar, but not identical way, since the rate of RecA*-mediated cleavage of UmuD is slower than that of LexA (98).

UmuD and UmuD′ can interact with UmuC in a variety of combinations. It is believed that the Umu(D′)$_2$C complex is active in the SOS mutagenesis being directly implicated in translesion synthesis. Bruck *et al.* showed that the purified

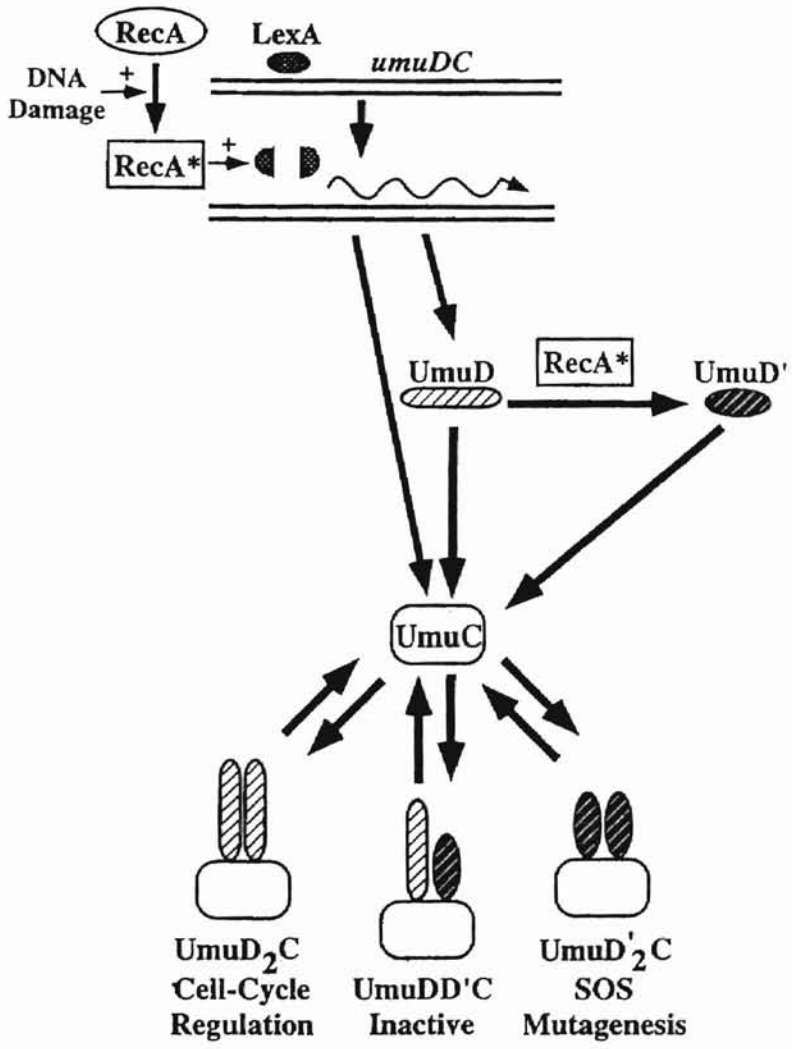
Umu(D')$_2$C complex was capable of binding to ssDNA, as well as to RecA-ssDNA nucleoprotein filaments (80). The biological consequences of the ssDNA binding activity are not clear yet but it is thought that could be involved in interactions with the DNA polymerase at the replication fork in the direction of the Umu proteins to damaged DNA (80).

The protein-protein interaction between UmuC and UmuD' has been shown using immunoprecipitation and yeast two-hybrid experiments. Also, there is evidence for the in vivo formation of UmuD'$_2$ and UmuD$_2$ homodimers.

The Umu(D)$_2$C complex seems to be involved in regulating the *E. coli* cell cycle after DNA damage while the UmuDD'C does not have an apparent activity, but may play a role in turning off SOS mutagenesis (80). In support of this theory, it has been shown that UmuD-UmuD' heterodimers form preferentially relative to the two homodimer species and inhibit SOS mutagenesis by tritrating out the active UmuD' (37). Rajagopalan *et al.* confirmed the inhibitory effect of UmuD on SOS mutagenesis in an *in vitro* translesion synthesis reaction (63). The association between the UmuD'-UmuD heterodimer and *umuC* has been demonstrated by Woodgate *et al.* using affinity chromatography (99). Fig. 2 shows the different complexes that can be formed between UmuC, UmuD and UmuD' molecules.

The specific molecular mechanisms of *umuDC*-mediated translesion synthesis that lead to SOS mutagenesis are still not understood. Different models have been proposed to explain this process, including increase in DNA

Figure 2. Interaction of UmuD and UmuD' with UmuC. RecA* mediates the cleavage of the LexA repressor that results in the induction of the SOS genes including the *umuCD* operon. RecA* mediates also the cleavage of UmuD into UmuD'. UmuD and UmuD' can interact with UmuC in a variety of combinations. The Umu(D)2C complex seems to be involved in regulating the cell cycle after damage. The Umu(D')2C is active in SOS mutagenesis. The UmuDD'C complex does not appear to have an activity, but may play a role in shutting off SOS mutagenesis (80).


RecA
LexA
umuDC
DNA
Damage
+
RecA*
+
UmuD
RecA*
UmuD'
UmuC
UmuD2C
Cell-Cycle
Regulation
UmuDD'C
Inactive
UmuD'2C
SOS
Mutagenesis

polymerase processsivity and relaxation of the DNA polymerase's requirement for a proper Watson-Crick DNA structure for continued replication (80). Hagensee *et al.* have shown that only DNA polymerase III, the major replicative polymerase in *E .coli*, is implicated in SOS mutagenesis (32). Translation synthesis requires the presence of UmuC and UmuD' (or homologous proteins) as well as RecA, even when DNA polymerase III lacking the proofreading function is used (63). This observation suggests that suppression of the proofreading function of the DNA polymerase is not a mechanism by which translesion synthesis occurs.

Homologs of *umuDC*. Many chromosomal *umuD* and *umuC* homologues have been identified in many prokaryotes and eukaryotes. Numerous homologues of the *umuDC* operon have also been found on naturally occuring plasmids. The best studied of the homologues is the *mucAB* operon encoded by the conjugative plasmid pKM101. pKM101 is a derivative of the clinically-isolated, multidrug-resistant plasmid R46 (46). Because of its ability to increase susceptibility of cells to chemical mutagenesis, pKM101 was introduced into the Ames *Salmonella typhimurium* strains used for the detection of mutagens and carcinogens (56). Even though *S. typhimurium* carries two operons homologous to *umuDC*, the chromosmal $umuDC_{st}$ and the plasmid borne *samAB* operon, this bacterium is poorly mutable by UV (42, 57). UmuD and UmuC of *S. typhimurium* and *E. coli* can cross-complement and *S. typhimurium* mutagenesis can be boosted by additional UmuD activity. These results suggest that both of the *S.*

typhimurium operons are active *per se*, but their ability to promote UV mutagenesisis is partially or totally suppressed by unknown mechanisms (57).

A chromosomally encoded homolog of *umuC, dinB*, has been identified in *E. coli* (38). *dinB* has been shown to be involved in mutagenesis since a mutant strain was found to be defective in the untargeted UV mutagenesis of bacteriophage λ. Furthermore, overexpression of the protein enhanced mutagenesis of an F'*lac* plasmid (41). In contrast to *umuC, dinB* does not have an adjacent *umuD*-like partner. Interestingly, numerous DNA sequences encoding proteins with homology to UmuC and DinB have been found in many prokaryotic and eukaryotic systems leading to believe that the UmuC-like proteins family is an ancient one (80).

Another interesting homolog of the *umuDC* operon is the *impCAB* operon of the TP110 plasmid (42). This plasmid belongs to the incompatibility group IncI1 and encodes colicin production and immunity (42). This plasmid has been shown to encode homologs of the *umuD* and *umuC* called *impA* and *impB*, respectively. The *impA* and *impB* genes encode proteins of the similar sizes than the ones encoded by *umuC* and *umuD*, and their expression is under the control of the SOS regulatory system. However, and additional open reading frame, *impC*, precedes the other two genes in the *impCAB* operon. The *impC* gene is not absolutely required for the protection and mutation phenotype, and it is thought to perform a regulatory function (25).

The conjugative pherormone-responsive plasmid pAD1 of the gram-positive microorganism, *E. faecalis*, was shown to encode a UV-resistance determinant, called *uvr*. This determinant contained three ORFs designated *uvrA*, *uvrB* and *uvrC*. The *uvrA* gene encoded a 442-amino acid protein that showed 20% homology of the identical residues with the UmuC protein of *E. coli*. Comparison of the deduced amino acid sequence of *uvrB* and *uvrC* did not show any significant homology to any other reported protein (58). Mutational experiments showed that *uvrA* was not regulated by an *E. faecalis recA* and *lexA* like system but by *uvrC* (58).

Other homologs of the UmuDC system have been found in other Gram-positive bacteria. This includes the *din* (damage inducible) genes in *Bacillus subtilis* that form part of the SOS-like response in this microorganism (11).

Recently, Martin *et al.* (55) proposed the existence of a SOS-like system in *S. pneumoniae*. They have suggested that the *recA* gene of *S. pneumoniae* is under the control of a LexA-like repressor protein. Consistent with such a model is the observation of RecA-dependent lysogenic induction following mitomycin C treatment suggesting that the RecA protein controls prophage induction by DNA damaging agents. On the other hand, the observation that many isolates of *S. pneumoniae* lacked error-prone repair following UV irradiation and thymine starvation (27) has led to propose that this microorganism is naturally devoid of the UmuDC-like functions of the SOS response (55).

CHAPTER III

MATERIALS AND METHODS

Bacterial Strains and Plasmids

Strains of *E. coli* used in this study are described in Table 1. Recombination deficient *E. coli* were used for amplification and purification of recombinant plasmids.

The non-encapsulated laboratory strain equivalent to wild type *S. pneumoniae* used in this study is Rx1 (78). Other pneumococcal strains were derived from Rx1. Strain SP1000 (5) is a Rx1 derivative that carries Tn*5252* in the chromosome. Strain SP1265 is a novobiocin-resistant derivative of SP1254. SP1254 was created by directed insertion of *E. coli* plasmid, pVA891 (53), into Tn*5252* in SP1000 (39). Insertion of pVA891 in SP1254 and SP1265 does not affect the transfer abilities of Tn*5252* (Tra^+). Therefore, SP1265 was used as a control in conjugation experiments.

The *S. pyogenes* strain, ATCC19615, used for conjugation was kindly provided by the Stillwater Medical Center. The UV-sensitive strain of *E. faecalis,* UV202 (100), was used in the preliminary experiments. SF5002 is a derivative of

Table 1

E. coli Strains

Strain	Relevant characteristics	Reference or Source
DH5α	Φ80*lacZ*ΔM15, *recA*1, *endA*1, *gyrA*96, *thi-1*, *hsd*R17 (r_K^-, m_K^+), *supE*44, *relA*1, *deoR*, Δ*lacY*U169	Bethesta Research Laboratories
JM109	*recA*1 *supE*44 *endA*1, *hsd*R17 *gyrA*96 *relA*1 *thiA*, (lac-*proAB*) (F' *traD*36 *proAB* + *lacI*^{q}*lacZ*ΔM15)	(101)
AB1157	*umu*$^+$ F$^-$ *thr-1 leu-6 proA*2 *his-4 thi-1 argE*3 *lacY*1 *galk*2 *ara-14 xyl-5 mtl-1 tsx-33 rpsL*31 *supE*37 λ$^-$	(43)
RM1140 or GW3189	Same as AB1157, but *umuC36*	R. V. Miller

UV202 carrying a copy of Tn*5252* inserted in the chromosome (5). Pneumococcal and other streptococcal strains used in this study are shown in Table 2.

Cloning vectors and plasmids used in this study are listed in Table 3. The plasmid, pAT29, is a shuttle vector conferring resistance to spectinomycin used for molecular cloning in *E. coli* and in Gram-positive bacteria (91). The plasmid, pVA891, can replicate autonomously and confers resistance to erythromycin and chloramphenicol in *E. coli*. When inserted into the streptococcal chromosome, it expresses only erythromycin resistance. It is incapable of autonomous replication in streptococci (53).

Growth Conditions

E. coli strains were grown at 37°C in Luria-Bertani (LB) broth supplemented with appropriate antibiotics, when needed, to maintain recombinant plasmids.

Strains of *S. pneumoniae* were routinely grown in CAT broth containing 1.0 % casein hydrolysate, 0.5 % tryptone, 0.1 % yeast extract, and 0.5 % NaCl. Sterile CAT broth was supplemented with 0.5 % (w/v) glucose and 15 mM K_2HPO_4 before use. Pneumococcal strains were grown at 37°C without aeration

Table 2

Streptococcal Strains

Strain	Relevant characteristics	Reference
S. pneumoniae		
RX1	*hex*	(78)
DP1100	*hex* UV^SI	W. R. Guild
DP1110	*hex* UV^SII $mitC^S$	W. R. Guild
DP1112	*hex* UV^SI+II	W. R. Guild
DP1617	*str-1 ery-2 nov-1 fus sulf*-d	(82)
SP1000	*str-1* fus Tn*5252*(*cat*)	(5)
SP1254	*str-1* fus Tn*5252*(*cat*ΩEm) Tra^+	(40)
SP1264	*nov-1* Tn*5252*(*cat*)	(70)
SP1265	*nov-1* Tn*5252*(*cat*ΩEm) Tra^+	(70)
SP1291	*str-1* Tn*5252*(*cat*ΩEm)	This study
SP1308	*str-1* UV^SI	This study
SP1311	*str-1* UV^SII $mitC^S$	This study
SP1314	*str-1* UV^SI+II	This study
SP1317	*str-1* pSJ142(Spc^r)	This study
SP1319	*hex* pSJ142(Spc^r)	This study
SP1323	*str-1* pAT29(Spc^r)	This study
SP1324	*str-1*(pSJ142 Δ 0.5 kb *Xba*I) (Spc^r)	This study
SP1400	*nov-1* Tn*5252*(*cat*ΩEm)	This study
SP1402	*str-1* Tn*5252*(*cat*)	This study
SP1405	*str-1* Tn*5252*(*cat*ΩEm)	This study
S. pyogenes		
ATCC19615	*opt*	Stillwater Med. Cent.
E. faecalis		
JH2-2	Wild type, plasmid free	(100)
UV202	*fus rif* UV^S	(100)
SF5002	*fus rif* Tn*5252*(*cat*)	(5)
SF5004	*fus rif* pSJ142(Spc^r)	This study

Table 3

Plasmids

Plasmid	Relevant characteristics	Reference or Source
pAT29	6.7-kb *E. coli-Streptococcus* shuttle vector; *lacZ* Spc^r	(91)
pBluescriptSK(+)	2.9-kb *E. coli* cloning vector; *lacZ* Amp^r	Stratagene
pBluescriptKS(+)	2.9-kb *E. coli* cloning vector; *lacZ* Amp^r	Stratagene
pVA891	5.9-kb *E. coli* cloning vector; Cm^r Em^r	(53)
pDR6	4.55-kp *Eco*RI fragment from Tn*5252* cloned into pBluescriptKS(+)	This study
pSJ142	4.55-kp *Eco*RI fragment from Tn*5252* cloned into pAT29	This study
pUM103	2.5-kb *Hind*III/*Eco*RI fragment from Tn*5252* cloned pBlueScriptKS(+)3.3-kb	This study
pUM104	0.5-kb *Xba*I fragment from Tn*5252* cloned into pBlueScriptKS(+)	This study
pUM105	2.5-kb *Xba*I fragment from Tn*5252* cloned into pBlueScriptKS(+)	This study
pUM106	4.8-kb Exonuclease/S1 derivative of pUM103	This study
pUM107	4.2-kb Exonuclease/S1 derivative of pUM103	This study
pKM101	34.5-kb plasmid derivative from plasmid R46; carries the *mucAB* operon	(56)
pSE117	10-kb plasmid derivative of pBR322,carries the *umuCD* operon of *E. coli*	(54)

to an OD_{550} of 0.2 (ca. 2 X 10^8 CFU/ml) and used immediately or stored at –80°C in 10% (v/v) glycerol.

S. pyogenes and *E. faecalis* strains were grown at 37°C in CAT broth without aeration for a minimum of 16 hours and stored at -80°C in 10% (v/v) glycerol.

For solid medium, 1.5 – 2.0 % agar was added to the broth. *E. coli* strains were plated on LB agar plates. *S. pneumoniae* was plated on CAT agar supplemented with 4% bovine blood using the agar-overlay technique to provide anaerobic conditions (78). *E. faecalis* and *S. pyogenes* strains were plated on the surface of CAT agar plates.

Antibiotic supplements used for selection of bacteria are listed in Table 4.

Chemicals, Restriction Endonucleases, and Media

Bacteriological agar and media were purchased from Difco. All chemicals and antibiotics were obtained from Sigma Chemical Co. Restriction endonucleases, T4 ligase, labeling kits and modifying enzymes were supplied by United States Biochemical (USB), New England Biolabs (NEB), Promega Corp., Bethesda Research Laboratories (BRL), or Boehringer Corp., and used as described by the manufacturer. Agarose was purchased from Fisher or Bio Rad Laboratories. Radionucleotides were obtained from New England Nuclear Co. (NEN) or Amersham Co.

Table 4

Antibiotics and Concentrations

Phenotype	Antibiotic	Concentration (µg/ml)	
		Stab Pate	Overlay
E. coli			
Cm^r	chloramphenicol	10	
Tc^r	tetracycline	10	
Em^r	erythromycin	200	
Km^r	kanamycin	50	
Ap^r	ampicillin	50	
Spc^r (pAT29)	spectinomycin	150	
S. pneumoniae			
cat	chloramphenicol	5	15
Em	erythromycin	3	5
Fus	fusidic acid	10	50
Nov	novobiocin	10	10
Rif	rifampicin	10	10
Str	streptomycin	200	200
Tet	tetracycline	2	5
Spc^r (pAT29)	spectinomycin	200	350
*aphA (*Tn*1545)*	Kanamycin	500	1000
E. faecalis			
Cm^r	chloramphenicol	25	
Fus^r	fusidic acid	25	
Rif^r	rifampicin	25	
Str^r	streptomycin	200	
Tc^r	tetracyclin	4	
Spc^r	spectinomycin	250	
S. pyogenes			
cat(Tn*5252*)	chloramphenicol	5	
Em^r	erythromycin	5	
Opt	optochin	20	

Molecular Cloning Techniques

DNA digestions, ligations and agarose gel electrophoresis were performed as outlined by Sambrooks *et al.* (69). For insertion mutagenesis in *S. pneumoniae*, the insert:vector ratio was 1:1 in a 60 µl volume. After overnight ligation at 16°C, the ligation mixture was extracted with phenol-chloroform, linearized with the appropriate restriction enzyme and then used as donor DNA in transformation of competent pneumococcal cells.

Agarose Gel Electrophoresis

DNA samples were prepared with an appropriate amount of tracking dye (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol, 20% glycerol, 0.1 M EDTA, pH 8.0) and loaded onto the gel along with a molecular weight standard marker. Electrophoresis was routinely carried out using 0.8% to 1.2% agarose gels and TBE buffer (89 mM Tris-base, 89 mM boric acid, and 2 mM EDTA, pH 8.0). After electrophoresis, gels were stained with 1.0 µg/ml ethidium bromide for 10 min and destained in deionized water for 10 to 20 min. DNA was visualized on the gels using an UV transilluminator and photographed using Alpha Imager 2000 (Alpha Innotech Corporation). For recovery of DNA fragments, samples were run in Ultra Pure DNA Grade agarose (Bio Rad) gels using the above conditions. Fragments were extracted and purified by

electroelution essentially as described by Sambrooks et al. (69)

Transformation

Transformation of *E. coli*

Competent cells were prepared and transformed with the relevant recombinant plasmids using the technique described by Hanahan (33). Transformant cells were isolated by plating appropriate dilutions of a transformation mixture on LB agar supplemented with the appropriate antibiotics. When necessary, IPTG (isopropyl-β-D thiogalactopyranoside) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D galactoside) were included in the agar for chromogenic differentiation of transformants.

Transformation of *S. pneumoniae*

Pneumococcal competent cells were prepared as described by Guild and Shoemaker (31). Aliquots of competent cells were stored at -80°C in 10% glycerol. Competent cells were thawed on ice, mixed with donor DNA (1 µg/ml for chromosomal DNA and 10 µg/ml for plasmid DNA) and incubated at 37°C for 30 min. DNaseI was then added to a final concentration of 10 µg/ml followed by incubation at 37°C for 5 min. Transformant cells were transferred to ice and

appropriate dilutions were plated on CAT agar using the overlay method.

***S. pneumoniae* Transformation Using Competence Factor.** The lyophilized preparation of competence factor was dissolved and diluted in 50 mM potassium acetate buffer (pH4.6) to a final concentration of 1µg/ml. To transform pneumococci, 500 µl of stored cells at an OD_{550} of 0.2 were mixed with 5ng of competence factor and transforming DNA. The cells were incubated at 37°C for 45 minutes. Plating was done as described for natural transformation.

Transformation of *E. faecalis*

E. faecalis cells were transformed by electroporation using a modified version of Fiedler's and Wirth's protocol (22). Cells were grown overnight in CAT broth. After harvesting the cells by centrifugation, they were washed twice in one volume of chilled 10% glycerol. After the second wash, the cells were washed again and suspended in 1/2 and 1/10 the original volume of chilled 10% glycerol. After the final wash, cells were suspended in 1/1000 the original volume in chilled 10% glycerol. 40 µl of cells and about 300ng of plasmid DNA were added to a chilled 0.2-cm electroporation cuvette and incubated for 5 min at 0°C. Cells were electroporated immediately in an Electro Cell Manipulator 600 (BTX Electroporation System) using the following settings: 1250 V, 25 µF and 200 Ω. After electroporation, cells were placed on ice for 1 to 2 minutes and then

transfered to 1 ml of CAT broth. Cells were incubated for 90 to 120 min at 37 °C for phenotypic expression of antibiotic resistances and then spread on selective CAT plates.

Plasmid Isolation from *E. coli*

Plasmid DNA screening in *E. coli* was performed by using the alkaline lysis method described by Sambrooks *et al* (69). Further purification of plasmid DNA isolated in this way was carried out by a second extraction with phenol-chloroform, followed by ethanol precipitation when required.

Large-scale isolation of plasmid DNA from *E. coli* was performed using cesium chloride-ethidium bromide density gradient ultracentrifugation. A crude lysate was obtained by following a scaled-up version of the protocol used for plasmid DNA screening. The lysate was mixed with cesium chloride and ethidium bromide and treated as described by Sambrooks *et al* (69).

Chromosomal DNA Isolation from *S. pneumoniae*

Broth cultures (200 ml) of pneumococcal strains were grown in CAT broth supplemented with 0.02% choline chloride. After reaching an OD_{550} of 0.3–0.4, cultures were mixed with 10 ml 10 mM EDTA and kept on ice for 10 min. Cells were washed twice with 50 mM Trish-HCl, 20 mM EDTA, pH 7.5 by centrifugation

at 5,000 x g in a Sorvall RC-5B centrifuge (DuPont Instruments). Cell pellets were resuspended in 5 ml of the same buffer followed by addition of 1 ml of a solution containing 0.6% triton X-100 0.06% sarkosyl, 0.6% sodium deoxycholate (DOC) and RNase (300 µg/ml). The cell suspension was incubated at 37°C until lysis was visible. Lysis was completed by adding 1 ml of 1% SDS and Proteinase K (350 µg/ml) and by overnight incubation at 65°C. DNA was purified by phenol-chloroform extraction and ethanol precipitation followed by drying and resuspension in 400 µl of TE buffer.

DNA Sequence Analysis

The nucleotide sequences were determined using PrismTM Ready Reaction DyeDeoxyTM Terminator Cycle Sequencing Kit, FS (Perkin Elmer) in the automated sequencer 373 Strech automatic sequencer (Perkin Elmer, Applied Biosystems) at the Recombinant DNA/Protein Resource Core Facility, Oklahoma State University.

Generation of Nested Deletions

To determine the sequence of the segments of interest, overlapping DNA fragments were obtained by generating nested deletions from fragments cloned in pBluescript SK+ or in pBluescript KS+ vectors. For this purpose the double

strand specific 3' to 5' exonuclease activity of the modifying enzyme Exonuclease III (USB) was used. The recombinant plasmids were digested with two different restriction enzymes in such a way as to generate a protected 4-base 3' end and a 5' overhang or blunt end adjacent to the insert from which deletions were to proceed. Digested DNA (ca. 5 µg) was mixed with 10 µl of 10X exonuclease III buffer (0.6 M Tris-HCl pH 8.0, 6 mM $MgCl_2$) and 450 units of exonuclease III enzyme. The reaction mixture was incubated on ice and 10 µl aliquots were removed every minute for 10 min. The samples were transferred to tubes containing 30 µl of S1 nuclease (BRL) (30 units in 10% glycerol, 60 mM NaCl, 1.3 mM $ZnSO_4$, and 8 mM potassium acetate pH 4.6) and held on ice until all samples were taken. Tubes were removed from ice and the reaction was allowed to proceed for 20 min at room temperature. The reaction was terminated by adding 4 µl of stop buffer (0.7 M Tris-Base pH 8.0, 50 mM EDTA) and by heat inactivating at 70°C for 10 min. Samples were purified with phenol-chloroform and chloroform-isoamyl alcohol followed by ethanol precipitation, self-ligated and used to transform *E. coli* competent cells. Transformants were screened for recombinant plasmids bearing inserts of smaller size and suitable for sequencing. When the partial sequences obtained from these clones were not sufficient to generate overlaps, synthetic oligomeric DNA primers were synthesized to complete the entire sequence on both strands.

Preparation of Double-Stranded DNA Template

Double-stranded DNA was prepared from overnight cultures of *E. coli* with the Magic miniprep kit (Promega Corp.) following the supplier's protocol.

Analysis of DNA sequences

Sequence data was assembled and analyzed by using MacVector v 4.5 (IBI), AssemblyLign (IBI) and DNASIS (Hitachi) software programs. Search for homologies was performed by comparing the deduced amino acid sequences to protein databases using the BLAST Network Service at NCBI, via internet. Bestfit analyses of the deduced amino acid sequences of potential open reading frames were performed by using the UWGCG Sequence Analysis Software Package (Genetic Computer Group, Inc. Wisconsin) via the University of Oklahoma Computer Center.

Southern Hybridization

Southern transfer of DNA on 0.8% agarose gels to GeneScreen Plus membranes (DuPont, NEN Research Products) was performed using a protocol of Southern (85) and according to the manufacturer's directions. Prior to transfer, DNA was denatured by soaking the gel in 0.5 N NaOH for 30 min.

Probes were radioactively labeled with (α-^{32}P)dCTP (Amersham Corporation) by nick translation following the protocol described by Sambrooks et al. (69). The probe concentration was adjusted to 1.0 to 4.0 X 106 dpm for each hybridization to obtain significant signal over noise. After hybridization at 65°C for 18 hours, membranes were washed, dried and exposed to X-ray film (Kodak BioMax MS) at -80°C for an appropriate time period.

Conjugation

Conjugation and scoring for trasconjugants between pneumoccal donors and recipients was performed essentially as described by Smith and Guild (84). Broth cultures of donor and recipient strains were grown to a density of 2 X 10^8 CFU/ml in CAT medium supplemented with 0.001% choline chloride and no selection. Donor and recipient cells were mixed at a ratio of 1:5 (donors:recepients) in the presence of 10 mM $MgSO_4$, 2 mg/ml bovine serum albumin (BSA), 1 mM $CaCl_2$, 0.2% yeast extract, and 1 mg/ml DNase I. About 1 X 10^9 cells were passed through nitrocellulose filters (pore size: 45µm, Millipore). Filters were then placed cell-side-down onto 2% CAT agar containing 10 mM $MgSO_4$, 2 mg/ml BSA, 1 mM $CaCl_2$, 0.2% yeast extract, and 100 µg/ml DNase I. Filters were overlaid with 6 ml of the same agar and the plates were incubated for 4 h at 37°C. After incubation, the filters and surrounding agar were removed and washed in 3 ml of CAT broth containing 10 mM $MgSO_4$, 2 mg/ml BSA, 150

µg/ml DNase I, and 10% glycerol. The cell suspension was vortexed thoroughly to remove cells from filters and serial dilutions were plated on selective media using the agar overlay method. Prior to the addition of medium containing antibiotics, plates were incubated at 37°C for 90 min to allow for phenotypic expression. The frequency of conjugation was calculated by dividing the number of transconjugants per ml by the number of donor cells present after the mating.

Essentially, the same procedure was used in conjugations betwen pneumococcal donors and *S. pyogenes* or *E. faecalis* recipients except that the conjugation was allowed to continue for 18 hours. *E. faecalis* and *S. pyogenes* transconjugants were scored by direct plating on the surface of selective media rather than by the agar overlay method used to score pneumococcal transconjugants.

Sensitivity to UV irradiation

Cells were grown to early exponential phase (approximately 10^8 CFU/ml) in the appropriate broth at 37°C. Cultures were harvested by centrifugation at 5000 g in a Sorvall RC-5B centrifuge (DuPont Instruments). The cells were washed twice and suspended in chilled PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH7.4). 3 ml of cell suspension was placed in a petri dish (60 x 15 mm) and exposed to various doses of UV radiation from a germicidal bulb (Model XX-15F, Spectronics Corporation) with a peak

emission of 256 nm. Doses of UV radiation were determined with a UVX radiometer (Ultra Violet Products) Survivors were determined by plating appropriate dilutions of cells on agar plates. LB plates were used for *E. coli* cells while CAT plates were used for *E. faecalis* cells. For pneumococcal cells, the overlay technique was used. All manipulations and incubations subsequent to UV irradiation were carried out in the dark or under amber light in order to minimize photoreactivation.

Chromosomal Mutagenesis

In *E. coli*

Overnight cultures in LB broth were diluted 1:50 in LB broth and grown with agitation to a concentration of about 2 x 10^8 CFU/ml. Then, cells were centrifugated and suspended in chilled PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4, adjusted to pH7.4). Samples were irradiated as described above and immediately diluted in 1:10 LB broth. Cells were grown in the dark for about two hours to allow some doublings to take place before selection was exerted. Reversion to histidine prototrophy was assayed by plating 250 µl, 100 µl and 10 µl samples on His^- plates containing Davis and Mingioli salts (3) plus glucose 4% (w/v), proline, threonine, valine, leucine and isoleucine (all at 100 µg/ml); 0.25 µg/ml thiamine and 1.5% (w/v) agar. The percentage of

survival was determined by plating dilutions of the irradiated culture on plates containing all the components mentioned above plus 100 µg/ml of histidine (79).

In *S. pneumoniae*

UV-treated *S. pneumoniae* cells were diluted 20-fold in CATPG medium, grown to 2 x 10^8 CFU/ml at 37°C in the dark, and plated on appropriate selective plates to score for survivors and drug resistant mutants. When needed, the cells were stores at -80°C in 10% glycerol. The following day, the cells were thawed in an ice-water bath, diluted and plated.

CHAPTER IV

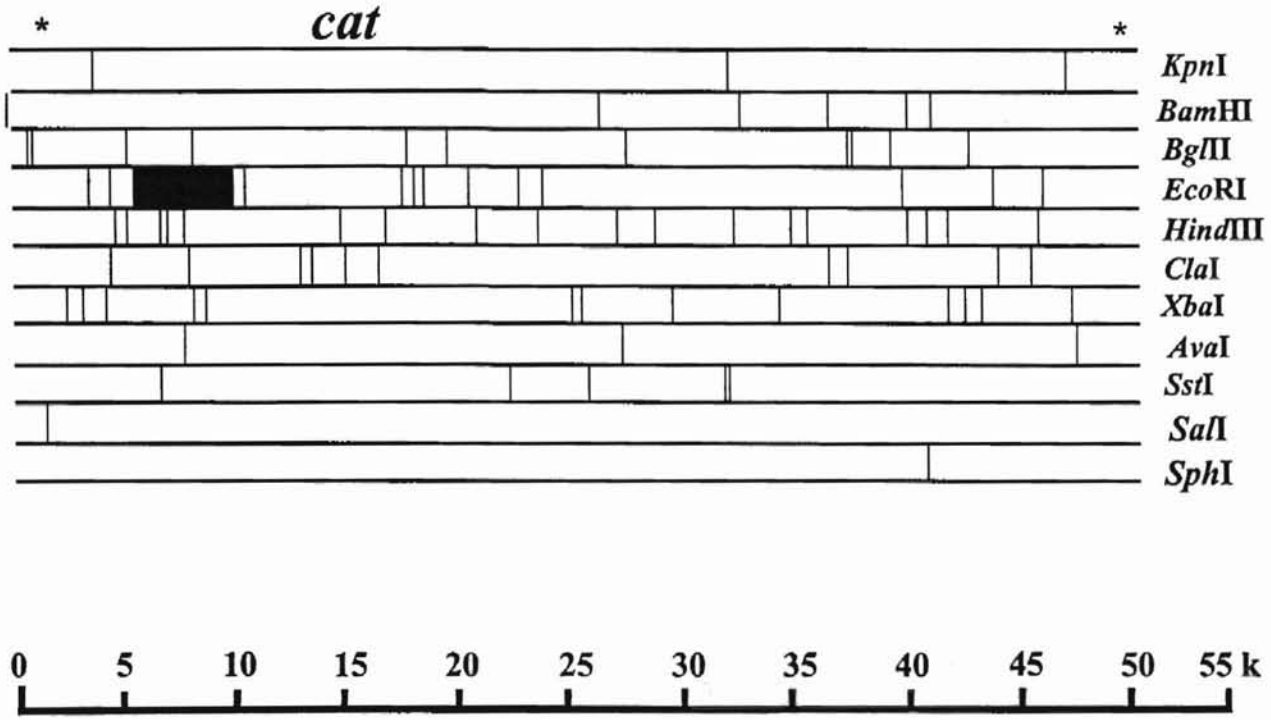
GENETIC AND TRANSCRIPTIONAL ORGANIZATION OF A REGION CARRING A UV-LIGHT RESISTANCE OPERON

RESULTS AND DISCUSSION

Identification and Nucleotide Sequence Analysis

Previously, it had been shown that the genes encoding DNA processing functions such as site-specific recombination and DNA relaxation during conjugal transfer of Tn*5252* were present at the left terminal region of the element (40, 86, 87). To identify other possible transfer-related genes nearby, the DNA sequence of about 2.5 kb of DNA on the right side of the DNA relaxase, and within a 4.55 kb *EcoR*I fragment, was determined. This segment is located between coordinates 8 and 10.5 in the restriction map of Tn*5252* shown in Fig. 3.

Figure 3. Restriction endonuclease map of Tn*5252*. The region of interest for this study is highlighted. The asterisks mark the ends of the element.


*
cat
*
KpnI
BamHI
BglII
EcoRI
HindIII
ClaI
XbaI
AvaI
SstI
SalI
SphI
0
5
10
15
20
25
30
35
40
45
50
55 k

Sequence Analysis

Construction of Recombinant Plasmids for Sequencing. For DNA sequencing, a 4.55 Kb *Eco*RI fragment, located between coordinates 5.95 and 10.5 and previously cloned into pKS(+) to create pDR6, was used. Sequence of the first 2 kb of DNA, located between coordinates 5.95 and 8 kb, had been obtained previously (86, 87). To determine the complete nucleotide sequence of this segment of DNA, subclones containing segments from the 4.55 kb *Eco*RI fragment were created. The recombinant plasmid pUM103, carrying DNA from coordinates 8 to 10.5, was constructed by digesting pDR6 with *Hin*dIII. After digestion, the larger *Hin*dIII fragment was electro-eluted and self-ligated to yield pUM103. To subclone the 0.5 kb *Xba*I fragment between coordinates 8 and 8.5 of the transposon, pDR6 was digested with *Xba*I. The 0.5 kb fragment was isolated and cloned into pKS(+) to create pUM104. The recombinant plasmid, pUM103, was digested with *Xba*I resulting in two fragments due to the presence of a unique site in the passenger DNA and another in the polylinker region of the vector. The 2 kb fragment, containing the transposon DNA from coordinates 8.5 to 10.5, was isolated and cloned into pSK(+) to create pUM105. The recombinant plasmid, pVJ545, containing a 0.7 kb *Hin*cII fragment containing DNA from coordinates 8.3 to 9, was also used in sequencing reactions to connect and complete the sequence.

In addition, a series of overlapping DNA fragments was obtained by

exonuclease III and S1 nuclease treatment of pUM103. The plasmids were sequenced using the M13 universal and reverse primers. Additional synthetic primers were designed, when necessary, to complete the sequence of both strands.

Sequence Features. The physical organization of the fragment of interest and the sequencing strategy used are shown in Fig. 4.

After assembly and analysis of the sequences from different DNA fragments an operon-like structure was found. This operon-like structure was about 3230 nucleotides in length. The G+C content of this region was 33.6% as expected for streptococcal DNA. Transcription of the potential ORFs proceeds from right to left (Fig. 4). The complete sequence with the orientation of transcription and the translated ORFs is shown in Fig. 5. Nucleotide 1 starts at coordinate 10.5 and proceeds to the end of the segment to coordinate 7.2 in the Tn*5252* restriction map.

Nucleotide Sequence Organization

The complete sequence of the 3.2 kb fragment revealed the presence of four ORFs, ORF14, ORF13, ORF12 and ORF11. The four ORFs were found to be transcribed in the same orientation as a single unit with a putative ribosome-binding site (RBS) placed upstream of each at an appropriate distance. A Gram-

Figure 4. Restriction map and predicted gene organization of the UV-resistance operon of Tn*5252*. The *Eco*RI site at the right end is about 8.5 kb from the left end of the element. Relevant restriction sites are shown. Thin line, chromosomal DNA; box, transposon DNA; crosshatched box, the 4.5-kb DNA containing the UV resistance genes; black boxes, direct repeat of IS-like sequences. The location of *cat* is shown. The vertical lines in the transposon indicate *Eco*RI sites. The direction and length of the potential ORFs are shown. Subclones derived from the 4.5-kb *Eco*RI fragment and a nested set of deletion derivatives obtained following exonuclease and S1 treatments were used to determine the sequence data from both strands.

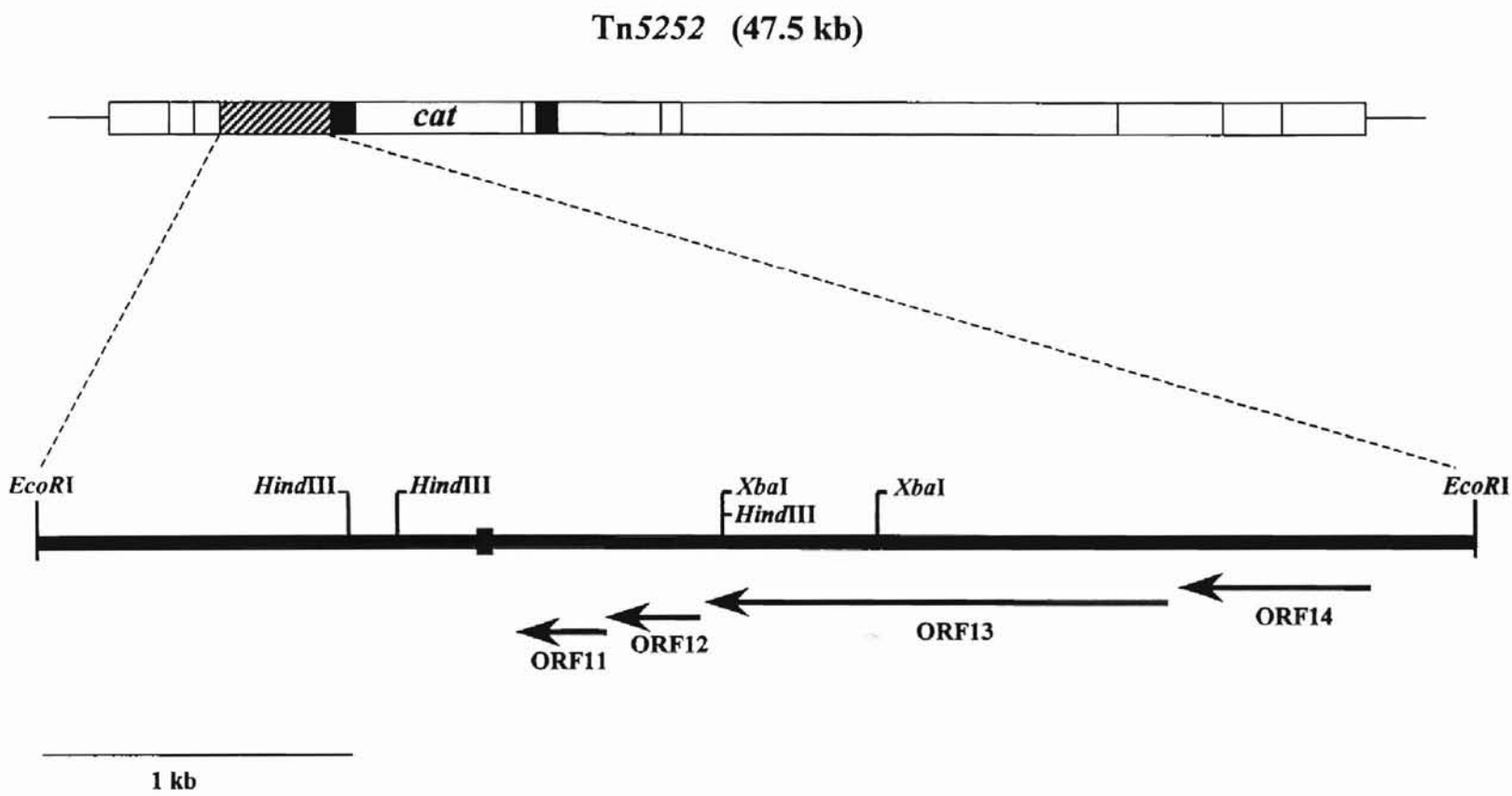

Tn5252 (47.5 kb)
cat
EcoRI
HindIII
HindIII
XbaI
HindIII
XbaI
EcoRI
ORF11
ORF12
ORF13
ORF14
1 kb

Figure 5. Complete nucleotide sequence of the 3.2-kb fragment fromTn*5252*. Translated sequences from ORFs are indicated. Long arrows indicate inverted imperfect repeats.

*Eco*RI

```
   1  GAATTCAAATTTACCAACGAGATAGAGGTTGTGTATATGATGCAAAAGAAAATACAACTT   60

  61  CAGCGGCAGATGTTCTTCAAGAGTTACTGTTTGGGGAGTGGAGTCAAGTAGAGAAGGATA  120

 121  TGCTTAAATCTGGAGAAGAAAGATTGAAAGATTTAACTAATCGAAATGGTTGTTAGATCA  180

                                    IR1
                                 ------>  <------
 181  TGAGTTTTAAAATATAGTCTATTGGATCGTCATTTTAAAAATAACGATCCTTTTTTTTAT  240

 241  TTTCTATTTTTGCTTGTTCTTTATCTTTTTCAAAAGTTATAACTTTCTTAAGTGATGGTT  300

 301  TCCTAGCAATATAAGTACAAATTAAGCACAGTTTATGATTATTTTAAATCTTGGGGGTTA  360

                                                IR2
                                 ------------------------>  <------
 361  TTAAACTCTTGATTTTTTCAATAAGCTATGGTATCCTTTTGGTAGTTAATAAACTTACAA  420

      ----------
 421  AAGGAGAACAAATGGTTTCACCTACTAAATTAACAGGAAAAAAAGAGAAGGCCAAGGATTA  480
   1     ORF14>  M  V  S  P  T  K  L  T  G  K  K  R  R  P  R  I  I   17

 481  TCTCAATCTCACTTGCATCCAGTTGGGAAATAATAGATCTTCAACTATTTAAACCTTTCA  540
  18    S  I  S  L  A  S  S  W  E  I  I  D  L  Q  L  F  K  P  F  T   37

 541  CAAACCCTAATCAAAATAATCTAAGCAAATTGAGTGAGATTTTGAATGTAGACCCTCGAT  600
  38    N  P  N  Q  N  N  L  S  K  L  S  E  I  L  N  V  D  P  R  Y   57

 601  ATTTTGAATCAGAGTATGAAATAGTACAAACTTATCTCAAACTAACGGAAAGGAATCAAG  660
  58    F  E  S  E  Y  E  I  V  Q  T  Y  L  K  L  T  E  R  N  Q  E   77

 661  AAGCAACACTTCATTATGCTACAGAACTATTGAACAAACAGAATGCGAAGGTTGTAGAAA  720
  78    A  T  L  H  Y  A  T  E  L  L  N  K  Q  N  A  K  V  V  E  I   97

 721  TTCCTGAGCGTTTTGCTTATAAAGTTTACGAAAAATTATCAGCTGGTACAGGAACAGCTT  780
  98    P  E  R  F  A  Y  K  V  Y  E  K  L  S  A  G  T  G  T  A  Y  117

 781  ATTTTGATGATGGTAATTACGATACAGTTTATTTTAATCATCAATTTGATTATGACTTTG  840
 118    F  D  D  G  N  Y  D  T  V  Y  F  N  H  Q  F  D  Y  D  F  A  137

 841  CATCATGGGTGTTTGGAAATTCAATGGAACCGACATATGAAGATGGTTCTGTAGCCCTTA  900
 138    S  W  V  F  G  N  S  M  E  P  T  Y  E  D  G  S  V  A  L  I  157

 901  TTAAGCAAACGGGATTTGATTATGACGGGGCTATCTATGCCATAGATTGGGATGGTCAAA  960
 158    K  Q  T  G  F  D  Y  D  G  A  I  Y  A  I  D  W  D  G  Q  T  177

 961  CATATATTAAGAAAGTGTATCGTGAAGAAAATGGGCTTCGTTTAGTTTCACTCAATCGGA 1020
 178    Y  I  K  K  V  Y  R  E  E  N  G  L  R  L  V  S  L  N  R  N  197

1021  ACTATTCAGATAAGTTTGCGCCTTATGATGAGAATCCTCGCATTATAGGGAAAATAGTTG 1080
 198    Y  S  D  K  F  A  P  Y  D  E  N  P  R  I  I  G  K  I  V  G  217

1081  GGAACTTTATGCCTTTAGAGGACTAAGTATGAGTTGGTTTGATTATAGTCTCGAACCTCA 1140
 218    N  F  M  P  L  E  D  *  >                                   224

1141  AGAATGACATTGCCTTTATTGATATGAAATCTTTTTACGCAAGTGTAGAATGTGTAGATA 1200
```

```
1201  GAGGATTACACCCCACTTAAGACTTCGGCTTTGTGTTATGAGTCGTGCAGATAATTCTGC  1260
ORF13>  I  T  P  H  L  R  L  R  L  C  V  M  S  R  A  D  N  S  A    19

1261  GGGTTTAATCCTTGCTTCCTCTCCTATGTTTAAAAAGGTCTTTGGAAAGTCGAACGTTGG  1320
  20   G  L  I  L  A  S  S  P  M  F  K  K  V  F  G  K  S  N  V  G    39

1321  ACGTTCCTATGATTTACCATTTGATGTAAAGACTCGCAAATTCTCTTATTATAATGCTAA  1380
  40   R  S  Y  D  L  P  F  D  V  K  T  R  K  F  S  Y  Y  N  A  K    59

1381  GAAGCAAGGTTTACCGACAACGATAGACTATGTCCGCTATATAGAGGAATGGGCAAAATC  1440
  60   K  Q  G  L  P  T  T  I  D  Y  V  R  Y  I  E  E  W  A  K  S    79

1441  AACCGTGATTGTTCCCCGAGAATGGATACTTACCATTGCTGTCAACATGGAGATTCAAAA  1500
  80   T  V  I  V  P  R  E  W  I  L  T  I  A  V  N  M  E  I  Q  K    99

1501  AATCTTTCAAGATTTTGCAGCACCAGATGATATTTATCCCTACTCAATTGATGAAGGATT  1560
 100   I  F  Q  D  F  A  A  P  D  D  I  Y  P  Y  S  I  D  E  G  F   119

1561  TATTGATTTAACAAGTTCATTAAATTATTTTGTACCAGATAAAAGTATTAGTAGGAAAGA  1620
 120   I  D  L  T  S  S  L  N  Y  F  V  P  D  K  S  I  S  R  K  D   139

1621  TAAATTAGATATTATTTCGGCTGCTATTCAAAAAAAGATTTGGAGAAAAACTGGAATCTA  1680
 140   K  L  D  I  I  S  A  A  I  Q  K  K  I  W  R  K  T  G  I  Y   159

1681  TTCAACTGTAGGCATGTCTAATGCCAATCCCTTATTAGCTAAGCTGGCACTTGATAATGA  1740
 160   S  T  V  G  M  S  N  A  N  P  L  L  A  K  L  A  L  D  N  E   179

1741  AGCTAAAAAGACTCCGACAATGAGAGCCAATTGGTCCTATGAGGATGTCGAAAAGAAAGT  1800
 180   A  K  K  T  P  T  M  R  A  N  W  S  Y  E  D  V  E  K  K  V   199

1801  ATGGACTATTCCTAAAATGACAGATTTCTGGGGAATTGGAAATCGCATGGAGAAAAGATT  1860
 200   W  T  I  P  K  M  T  D  F  W  G  I  G  N  R  M  E  K  R  L   119

1861  ACATAATTTAGGTATCTTTTCGATTAAAGAATTGGCTCAGGCTAATCCTGACTTGATTAA  1920
 220   H  N  L  G  I  F  S  I  K  E  L  A  Q  A  N  P  D  L  I  K   239

                          XbaI

1921  AAAAGAGCTTGGTATCATGGGTCTAGAGTTATGGTTCCATGCCAATGGGATTGATGAAAG  1980
 240   K  E  L  G  I  M  G  L  E  L  W  F  H  A  N  G  I  D  E  S   259

1981  TAATGTTCATAAACCTTATAAGCCAAAGTCAAAAGGGATAGGGAACTCTCAAGTTTTACC  2040
 260   N  V  H  K  P  Y  K  P  K  S  K  G  I  G  N  S  Q  V  L  P   279

2041  AAAAGATTATATTAAACAAAGAGATATTGAGATCATACTTCGTGAGATGGCAGAACAAGT  2100
 280   K  D  Y  I  K  Q  R  D  I  E  I  I  L  R  E  M  A  E  Q  V   299

2101  TGCAGTTAGATTGAGAAGATCTGGTAAAAAAGCAACAGTAGTTTCTATACATCTAGGCTA  2160
 300   A  V  R  L  R  R  S  G  K  K  A  T  V  V  S  I  H  L  G  Y   319

2161  CTCTAAAGTGGAACAAAAGCGTTCTATCAATACTCAAATGAAAATTGAACCAACTAATCA  2220
 320   S  K  V  E  Q  K  R  S  I  N  T  Q  M  K  I  E  P  T  N  Q   339

2221  AACAGCACTACTGACAAACTACGTTTTAAAGTTATTTCACACTAAATATACTTCAGGAGC  2280
 340   T  A  L  L  T  N  Y  V  L  K  L  F  H  T  K  Y  T  S  G  A   359

2281  TATCAGGAATGTTGCGGTTAACTATTCTGGTTTAGTGGATGAATCCTTTGGATTGATTTC  2340
 360   I  R  N  V  A  V  N  Y  S  G  L  V  D  E  S  F  G  L  I  S   379

2341  ATTATTTGATGATATTGAGAAAATAGAAAAAGAAGAAAGGCTCCAGTCCGCAATCGATGC  2400
 380   L  F  D  D  I  E  K  I  E  K  E  E  R  L  Q  S  A  I  D  A   399
```

*Hin*dIII

```
2401  TATTCGAACAGAATTTGGGTTCACTTCACTATTGAAAGGTAATGCCCTAGATCAAGCTTC   2460
 400   I  R  T  E  F  G  F  T  S  L  L  K  G  N  A  L  D  Q  A  S    419
```

*Xba*I

```
2461  TAGAACAATTGCTAGAAGCAAACTCATTGGTGGTCATTCAGCTGGAGGATTAGATGGACT   2520
 420   R  T  I  A  R  S  K  L  I  G  G  H  S  A  G  G  L  D  G  L    439

2521  AAAATAATGAATGTTCTTATTTACCTTTTGAGTCTGCCCGAGTATATCAGGATGTGGCAT   2580
 440   K  *  >                                    ORF12>    V  A      2

2581  GGCTAAGTGGATGCTTCTTTTTATCAGAACATTCCAGTTCTCTTTGGGCAGAAAAAAATA   2640
   3   W  L  S  G  C  F  F  L  S  E  H  S  S  S  L  W  A  E  K  N     22

2641  AAGAAGATATCTCCATTTCCCTATCATTGGAAGAGAAAGTTCTATTTGTTCGTCAACTTT   2700
  23   K  E  D  I  S  I  S  L  S  L  E  E  K  V  L  F  V  R  Q  L     42

2701  ATACGAATGTATTCCCTGCAACTTTTGTTTTTAAGTTTTCTAATCAAAGAAAAGTAGTAT   2760
  43   Y  T  N  V  F  P  A  T  F  V  F  K  F  S  N  Q  R  K  V  V     62

2761  CAGGTATTGTTAAAGAGATTGGAAAAGAGTTTATATCTATAAAATCAGACACTGGTTTTC   2820
  63   S  G  I  V  K  E  I  G  K  E  F  I  S  I  K  S  D  T  G  F     82

2821  TTCGATTAAAATGGGAAGATATACTCGATATACAGATAGAAGGGGAGGAATTACATGAAT   2880
  83   L  R  L  K  W  E  D  I  L  D  I  Q  I  E  G  E  E  L  H  E    102
   1                                              ORF11>    M  N      2

2881  CGTAAAGAATTATATGATGATAAATTACAGCTAGATTATTTTTCAGATTCTTATTTACAG   2940
      S  *  >                                                        103
   3   R  K  E  L  Y  D  D  K  L  Q  L  D  Y  F  S  D  S  Y  L  Q     22

2941  TTTGAGTCAGATTTTTACAAGTATTCAGCTTTAGATATACCATTAACATTTATCACAGAT   3000
  23   F  E  S  D  F  Y  K  Y  S  A  L  D  I  P  L  T  F  I  T  D     42

3001  GATATTTTACGCACAATGGCTATGTCTCAAAAACATTATTTTAAACTTAACAAAAGTAAA   3060
  43   D  I  L  R  T  M  A  M  S  Q  K  H  Y  F  K  L  N  K  S  K     62

3061  TCTTTAGACGGTCGTGATCATTACTTTGTTTTTTCTATCAAGATGAACAAAGACAGTAGT   3120
  63   S  L  D  G  R  D  H  Y  F  V  F  S  I  K  M  N  K  D  S  S     82

3121  GGTATTAGACAGTATGAATATCAGAGACATTGTTTTAATTTGTAAGAGTCCGACAGGGCT   3180
  83   G  I  R  Q  Y  E  Y  Q  R  H  C  F  N  L  *  >                 96
```

IR3

```
3181  CTTTTTTCTGTGATAATTTTATCAAAAAGTATTTGTTATACTTTTTTTAATTTAGATTTA   3240

3241  TCTTGGGGGTTGGGGGGCGTGCCACCACATTTTCATATCGTTTGTTTTTTAGAACCGTGA   3300

3301  GGTTTGAAATGGCGGCGATATGATTTTTGGGATATTGTGGACACAATATCTGAGCTCGCA   3360
```

*Hin*dIII

```
3361  AAGCCATACAAAAATGTTGAATCTATTTTGTAAAACGTACTGACAGTGTATGTAAGCTT    3419
```

positive consensus promoter-like sequence (29) was noted about 140 bases upstream of the translational start-site of ORF14. Twenty-one bases upstream of the –35, a 14 bp inverted imperfect (12/14) repeat was present. In the RBS region of ORF14, another inverted repeat (17/21) with a ΔG value of –14.7 kcal/mol was located. ORF12 and ORF11, having the capacity to encode proteins with molecular masses of 11 kDa and 11.7 kDa, respectively, were found to overlap by eight bases.

ORF14and ORF13 could encode proteins with molecular masses of 26 kDa and 49.7 KDa, respectively. A summary of the DNA analysis of the 3.2 kb fragment is shown in Table 5.

Analysis of the ORFs Predicted Amino Acid Sequence

The characteristics of the predicted products in the 3.2 kb DNA fragment are summarized in Table 6. The deduced amino acid sequences of the putative ORFs were analyzed for similarities to other protein sequences in the GenBank. Amino acid sequences of ORF12 and ORF11 did not display significant similarity to any protein in the database.

Table 5

Properties of the Predicted ORFs in the 3.2 kb DNA Fragment

ORF	Size (bp)	Start Codon	Stop Codon
ORF11	291	ATG	TAA
ORF12	315	GTG	TAA
ORF13	1323	ATT	TAA
ORF14	675	ATG	TAA

Table 6

Characteristics of the Predicted Products in the 3.2 kb DNA Fragment

ORF	Amino Acid Residues	Molecular Weight (Da)	pI
ORF11	96	11	7.2
ORF12	104	11.7	4.9
ORF13	440	49.7	9.4
ORF14	224	26	4.8

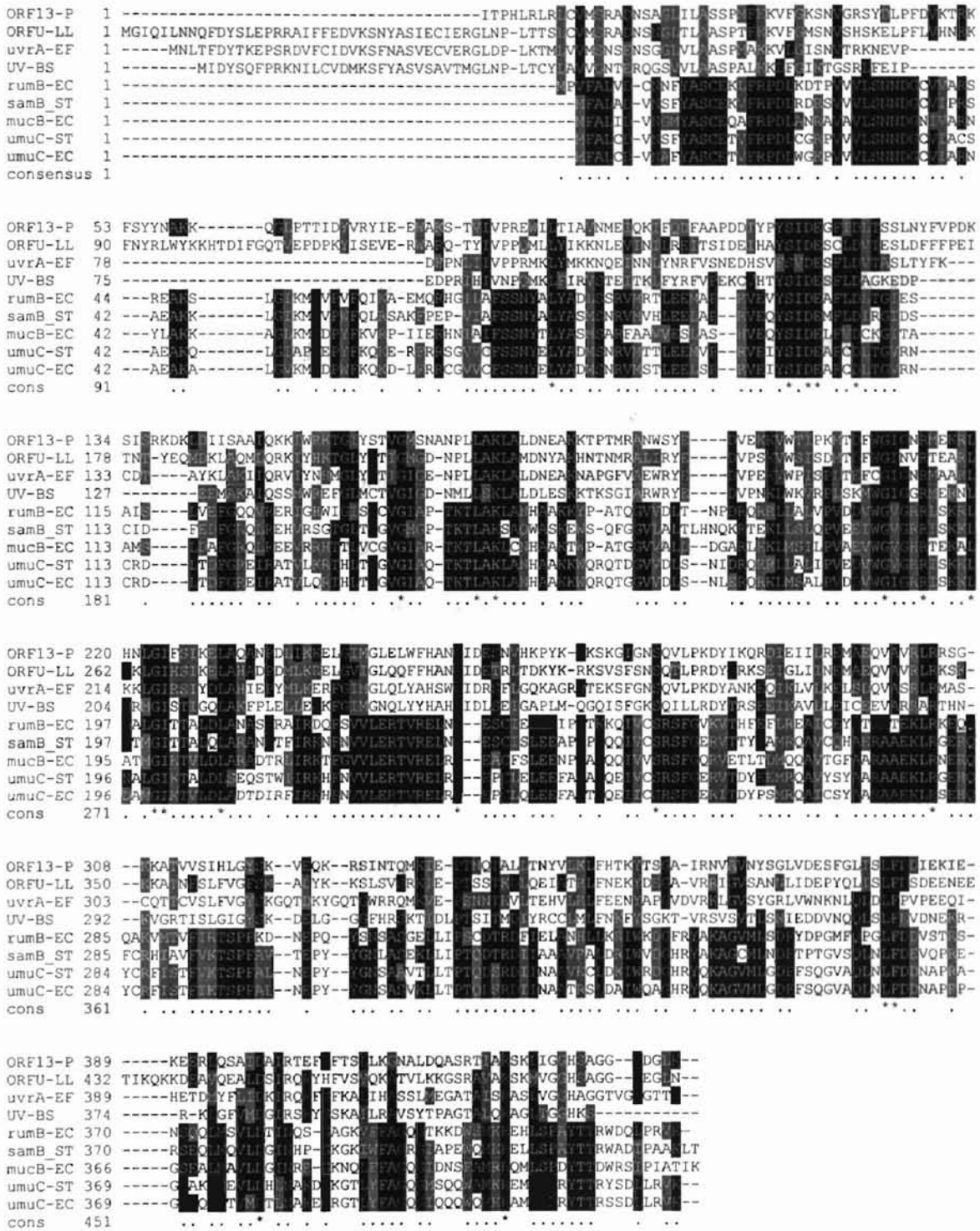
ORF13 Predicted Product

The deduced amino acid sequence of ORF13 was found to be a 49.7 kDa protein with a calculated isoelectric point of 9.44 (Table 6). The amino acid sequence of ORF13 showed highly significant similarity to proteins involved in the SOS response in Gram-positive and Gram-negative bacteria.

Multiple alignment between ORF13 predicted protein and the sequences with the highest level of homology is shown in Fig. 6. These included the UmuC of *E. coli* and *S. typhimurium* (80, 81), MucB produced by the plasmid pKM101 (56), their homologues in *B. subtilis* (11), and the UvrA protein encoded by pAD1, a pheromone-responsive plasmid from *E. faecalis* that confers increased UV-resistance to UV-sensitive strains (58). UmuC and MucB are proteins encoded by the *umuDC* and *mucAB* operons, respectively, which form part of the SOS regulon, and are necessary for the induced mutagenic response (80). The *umuDC* genes are chromosomally expressed while the mucAB genes are present on some conjugative plasmids (80) . The introduction of these plasmids into *E. coli* and *S. typhimurium* increased resistance to UV-light killing and susceptibility to mutagenesis (97).

The highest level of homology was between the predicted product of ORF13 and the predicted product of ORFU from the conjugative lactococcal phage resistance plasmid pNP40 of *L. lactis* subsp. *lactis* bv. diacetylactis DRC3 (52% identity, 63% similarity) (26). ORFU is a homolog of the *umuC* gene (26).

Figure 6. Multiple sequence aligment of the predicted product from ORF13 and its homologs. ORFU from *L. lactis* plasmid pNP10 (26), uvrA of *E.faecalis* (58), UV-damage repair protein of *B. subtilis* (accesion number Z99115), *rumB* of IncJ plasmid R391 from *E. coli* (44), *samB* from *S. typhimirium* (57), *mucB* of R46 from *S. typhimurium* (56), and the chromosomal *umuC* from *S. typhimurium* (81) and *E. coli* (59). Conserved amino acids are shaded in black while conservative substitutions are shaded in gray.



Interestingly, a *recA* homolog, designated *recALP*, was found in the proximity of ORFU (26).

ORF14 Predicted Product

The ORF14 deduced amino acid sequence was found to be a 26 kDa protein with a calculated isoelectric point of 4.8 (Table 5). Comparison of the amino acid sequence of ORF14 displayed high level similarity to a variety of transcriptional regulators of phages of Gram-positive bacteria. In particular, the similarity was significant around the three conserved domains in the RecA mediated cleavage of many of these proteins (59). Several other residues conserved in the LexA family of proteins and phage repressors (7) were also present in ORF14. The product of the gene *frp*, FRP, predicted to be the regulator of the fructosyltransferase expression is *S. mutans* (75), showed the highest level similarity to ORF14 (66% identity; 75% similarity). The similarity was very pronounced among the C-terminal residues of the protein. Fig. 7 shows the alignment of ORF14 with FRP from *S. mutans*. The conserved residues essential for RecA-mediated cleavage reaction are also shown.

Figure 7. Alignment of the predicted protein product of ORF14 and the product of the *frp* of *S. mutans*. Vertical bars represent identity and dots represent similarity. The conserved residues essential for RecA-mediated cleavage reaction are shown in bold.

```
 37 TNPNQNNLSKLSEILNVDPRYFESEYEIVQTYLKLTERNQEATLHYATEL 86
    |  |||  ||  ||. :|  ||   || |:::||: | :| | |.  ||  |.  |
 40 TKPNQKNLDKLAHLLKVDSAYFLSQHDIVEIYTRLNESNKTKTLKYSQHL 89

 87 LNKQNAK.VVEIPERFAYKVYEKLSAGTGTAYFDDGNYDTVYFNHQFDYD 135
    | .|. |  .   .|: |:||||||||| .|| |||:|||::. : |:|
 90 LEQQDKKRNLMKNKRYPYRVYEKLSAGTGYSYFGDGNFDTVFYDEEIDHD 139

136 FASWVFGNSMEPTYEDGSVALIKQTGFDYDGAIYAIDWDGQTYIKKVYRE 185
    ||||:||.||||  : .| ||||||||||||||||||||||||||||||
140 FASWIFGDSMEPIFLNGEVALIKQTGFDYDGAIYAIDWDGQTYIKKVYRE 189

186 ENGLRLVSLNRNYSDKFAPYDENPRIIGKIVGNFMPLE 223
    |  ||||||||: |.|||||||||||||| |||||.|||
190 ETGLRLVSLNKKYADKFAPYDENPRIIGLIVGNFIPLE 227
```

CHAPTER V

CHARACTERIZATION OF AN OPERON INVOLVED IN RESISTANCE TO UV-LIGHT AND SOS MUTAGENESIS

RESULTS AND DISCUSSION

UV-Induced Killing

Preliminary Studies Using *E. faecalis* Strains

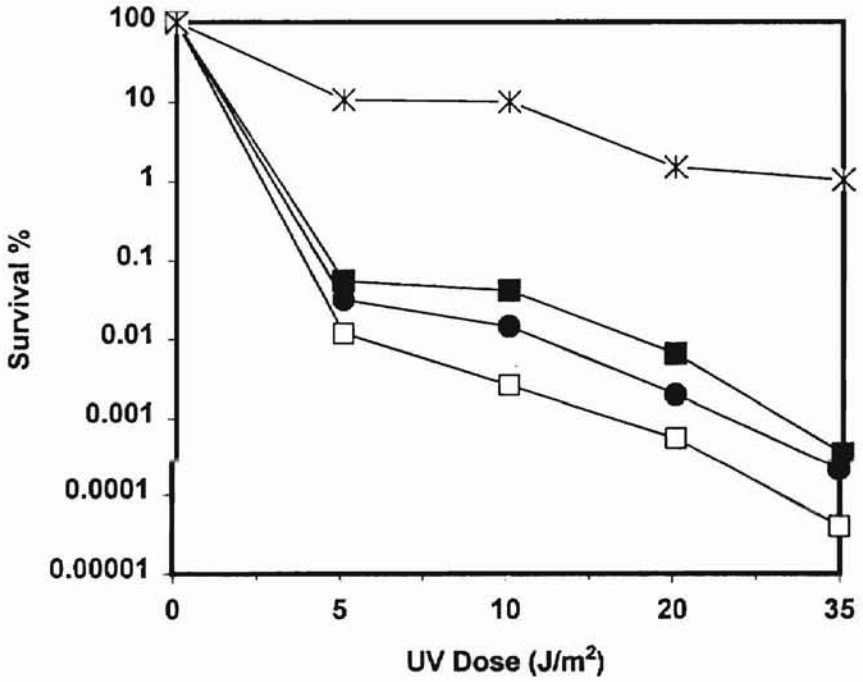
To determine whether the operon carrying ORF13 and ORF14 was involved in the enhancement of survival of the host cell upon UV-treatment, the 4.55 kb *Eco*RI fragment carring the region of interest was cloned into the *Streptococcus-E. coli* shuttle multicopy plasmid, pAT29, to create pSJ142. The recombinant plasmid was introduced into *E. faecalis* UV202 by electroporation. Spc^r colonies were isolated and the plasmid was recovered. *E. faecalis* UV202 carrying pSJ142 was designated SF5004. Also, SF5002 (5), a UV202 derivative containing a copy of Tn*5252* in the chromosome, was used in these experiments. *E. faecalis* UV202 is a derivative of *E. faecalis* JH2-2 (100) and has been shown

to be very sensitive to UV-light (100). The gene, which determines the UV sensitivity of *E. faecalis* UV202, has not been identified (58, 100).

Comparison of UV-sensitivity levels at various UV irradiation doses ranging from 5 to 35 J/m^2 showed an average of a four-fold increase in UV protection of SF5002 cells, carrying Tn*5252* with a single copy of the operon, in contrast with UV202 cells. These results indicated the involvement of the element in protection. On the other hand, UV resistance increased to about 10-fold in SF5004 cells carrying pSJ142 as compared to the cells without the plasmid. The copy number of pSJ142, a pAT29 based plasmid carrying the origin of replication of the broad host range enterococcal plasmid pAMβ1 (91), is seven. Therefore, the protection level of the cells carrying the recombinant plasmid was expected to be higher. Fig.8 shows the effect of UV irradiation on the survival of the different *E. faecalis* strains used in this study.

The results of this experiment showed a definitive role of the region of interest in UV protection. The presence of one or many copies of the UV resistance determinant of Tn*5252* clearly enhances the UV resistance of UV202. This, on the other hand, could suggest that UV202 is a mutant deficient in one of the genes involved in the SOS-like system of *E. faecalis.*

Figure 8. Effect of UV irradiation on survival of *E. faecalis* strains: -✳-, JH2-2; -□, UV202; -●-, SF5002; -■-, SF5004. The UV survival curves were obtained as indicated in Materials and Methods. The numbers represent the averages obtained from at least two independent experiments.


100
10
1
0.1
0.01
0.001
0.0001
0.00001
Survival %
0
5
10
20
35
UV Dose (J/m2)

UV-induced Killing in *S. pneumoniae* Strains

To study the effect of the UV-resistance determinant of Tn*5252* on *S. pneumoniae* strains, a number of pneumococcal strains were created.

Pneumococcal UV Sensitive Strains

Pneumococcal strains DP1100, DP1110 and DP1112 are UV-sensitive derivatives of Rx1 created by treatment with nitroso-guanidine, hydroxylamine, and both mutagenic agents, respectively (Walter R. Guild, personal communication). These strains were transformed with DP1617 DNA (*str-1 ery-2 nov-1 fus sulf-d*) in order to generate UV sensitive strains with *str* background as a selectable marker. The overlay method was used to select for streptomycin resistant transformants. The transformants were replica-plated on blood agar containing erythromycin and novobiocin, separately. Colonies that were resistant only to streptomycin were selected. The streptomycin-sensitive derivatives from DP1100, DP1110 and DP1112 were named SP1308, SP1311 and SP1314, respectively.

SP1308, SP1311 and SP1314 were exposed to different doses of UV irradiation (0, 5, 10, 25 and 50 J/m^2) to compare their UV sensitivity. SP1311 and SP1314 were noticeably more sensitive to UV-light than SP1308 (Fig.9). Since SP1311 is a derivative of DP1110, which was created by treatment with

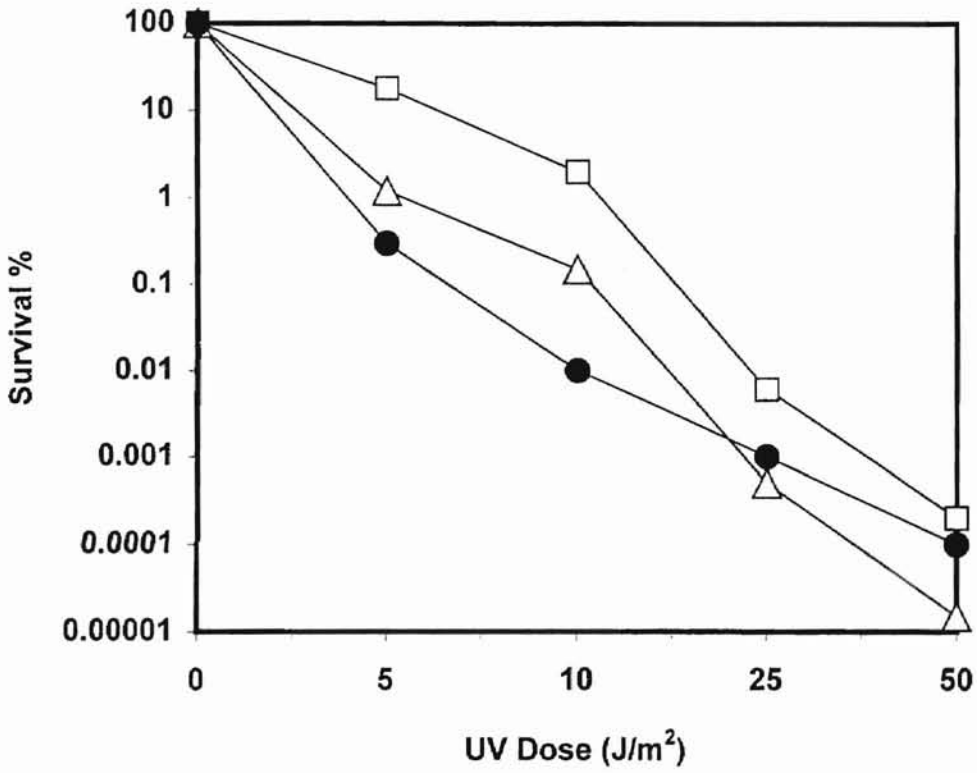

100
10
1
0.1
0.01
0.001
0.0001
0.00001
Survival %
0
5
10
25
50
UV Dose (J/m2)

Figure 9. Effect of UV irradiation on survival of *S.pneumoniae* -□-, SP1308; -△-, SP1311; -●-, SP1314. The UV survival curves were obtained as indicated in Materials and Methods. The numbers represent the averages obtained from at least two independent experiments.

hydroxylamine, this strain was selected to be used in the UV-induced killing experiments.

In vivo Deletion Mutation of ORF13

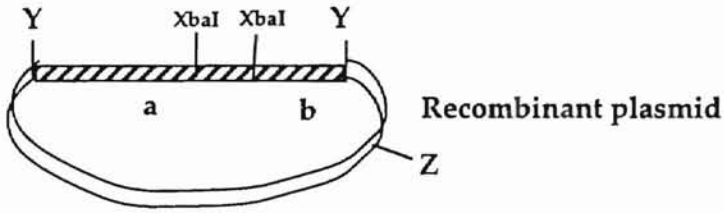
The homology between the predicted protein product of ORF13 and UmuC/MucB homologues of other systems suggested that this protein could also have a major role in UV resistance and UV-induced mutagenesis. In such a case, upon mutating the ORF13 locus, we would expect a decrease in UV resistance and induced mutagenesis. To test this hypothesis, a pneumococcal deletion mutant strain was created. For this, the plasmid, pVA891, was used to interrupt ORF13. The *E. coli* plasmid, pVA891, is not capable of autonomous replication in streptococci, but carries a streptococcal erythromycin resistance determinant that is expressed in pneumococci when the plasmid is integrated into the chromosome (53). The 0.5 kb *Xba*I fragment (Coordinates 8 to 8.5 of Tn*5252*) within ORF13 was removed from pSJ142 and replaced with *Xba*I-digested pVA891. The resulting ligated molecule was linearized with *Kpn*I that has a unique cleavage site at the vector, pAT29, part of pSJ142. The digested DNA was introduced into competent SP1000 cells, carrying Tn*5252* and *str-1* chromosomal point mutation, by transformation. Due to the flanking homology provided by the Tn*5252* DNA cloned in pSJ142, the heterologous pVA891 was expected to insert into the ORF13 locus on the chromosome in a homology-

dependent event. Fig. 10 gives a schematic representation of the insertion mutagenesis strategy used.

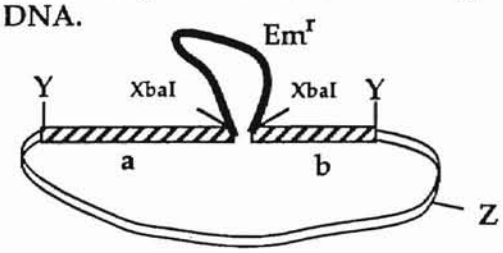
After transformation, the Em^r transformants were replica-plated on blood agar containing streptomycin. Chromosomal DNA from one of the resulting Em^r Str^r transformants, called SP1291, was analyzed by Southern hybridization using pDR6, carrying the 4.5 *Eco*RI fragment, as a probe to determine whether the insertion had taken place. SP1291, SP1000 and Rx1 DNAs were digested with *Cla*I and *Hin*dIII and run on the same gel and blotted on nylon membranes. The probe did not react with wild-type Rx1 cells, which did not carry Tn*5252*. Due to the presence of a single *Cla*I site within this region, the probe was expected to react with two fragments of 3.2 and 5 kb in SP1000 (Rx1::Tn*5252*). As expected, the probe reacted with two *Cla*I fragments of these sizes. In addition, the probe reacted with three *Hin*dIII fragments of 6.84, 1.34, and 0.98 kb of SP1000 DNA, the latter appearing more clearly in the original autoradiogram. Replacement of the 0.5 kb *Xba*I fragment with pVA891 in SP1291 was expected to result in the probe hybridizing to two (4.5 and 9.2 kb or 3.4 and 10.3 kb) *Cla*I fragments and three (1.34, 5, and 8.1 or 1.34, 2.8, and 10.3 kb) *Hin*dIII fragments depending upon the orientation of the insert. The probe reacted with fragments of the former group in each case, indicating the absence of any unexpected rearrangements (Fig. 11).

Figure 10. Strategy for creating a deletion mutant within ORF13 of Tn*5252*. A 0.5 *Xba*I fragment was deleted from a streptococcal plasmid carring the 4.5 *Eco*RI (Y) fragment (pSJ142). The larger fragment was ligated to the *E. coli* plasmid pVA891 carrying resistance to chloramphenicol and erythromicin. pVA891 is incapable of autonomous replication in streptococci and expresses erythromycin resistance when integrated into the chromosome. The resulting circle was linearized at a unique site within the vector (Z) and used as donor DNA to transform pneumococcal cells carrying Tn*5252*.

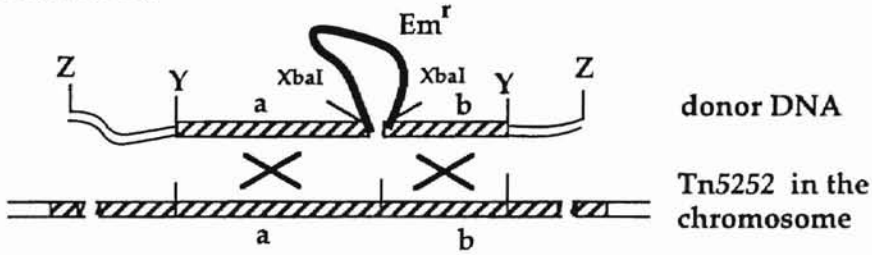
A - Isolate and ligate a fragment from the transposon to a suitable vector



B - Delete 0.5 Kb XbaI fragment and insert a drug resistance marker in the passenger DNA.



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



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

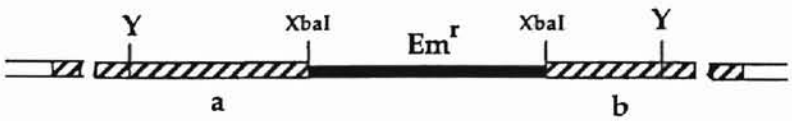
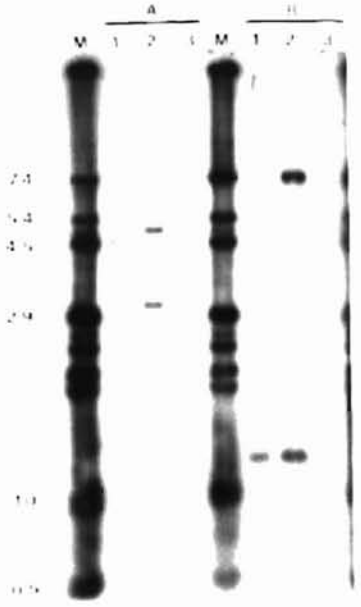


Figure 11. Physical analysis of Em^r transformants carrying the insertion of pVA891 within ORF13 in Tn*5252*. Autoradiogram showing Southern hybridization of ^{32}P-labeled pDR6 to *Cla*I (A) and *Hind*III (B) digested chromosomal DNA from (1) SP1291 (SP1000 carrying a deletion within ORF13), (2) SP1000 (Rx1::Tn*5252*), and (3) *S. pneumoniae* Rx1. The indicated sizes are for standards in lane M, consisting of a set of calibrated fragments from pSK(+) or derivative plasmids, all of which react with the probe.


A
M
1
2
3
M
1
2
3

Construction of SP1400. In order to obtain a strain containing the same mutation as in SP1291 but in a *nov*^{r} background, the strain SP1264, which carries a copy of *Tn5252* and is *nov*^{r}, was transformed with SP1291 DNA. After transformation, Em^r colonies were selected and replica-plated on blood agar containing chloramphenicol and novobiocin, separately. An Em^r, Cm^r, *nov*^{r} colony was chosen and designated SP1400.

SP1311 Derivatives

Construction of SP1317. The recombinant plasmid pSJ142, containing the fragment of interest, was introduced into SP1311 via transformation to create SP1317. After transformation the cells were plated using the overlay technique previously described. Transformants were selected for spectinomycin resistance and replica-plated on blood agar containing streptomycin. This strain was used to study the effect of multiple copies of the UV protection operon on the UV survival and induced mutagenesis response of the cells.

Construction of SP1323. The plasmid vector, pAT29, was introduced into SP1311 to create SP1323. This strain was used as a control in UV-killing and mutagenesis experiments. As with SP1317, transformants were selected for Spc^r and *str*^{r}.

Construction of SP1324. After deleting the 0.5-kb fragment from pSJ142, the larger fragment was religated and used for transformation of SP1311. Transformants were selected for Spc^r and *str*^{r}. This strain provides multiple copies of the plasmid with ORF13 mutated.

Construction of SP1402. After filter-mating, one true transconjugant (Em^r, Cm^r, *str*^{r} *nov*^{s}) from the SP1265 x SP1311 mating was chosen and designated SP1402. SP1265 carries a copy of Tn*5252* that has a pVA891 insertion in a region that does not affect transfer (*nov*^{r}, Cm^r, Em^r, Tra$^+$) (70). This strain, in contrast with SP1317, carries a single copy of the UV protection operon.

Construction of SP1405. The strain SP1405 was created the same way as SP1402, except that the donor strain for conjugation was SP1400, carrying the deletion mutation in ORF13.

UV-Induced Killing

In the highly UV-sensitive pneumococcal strain, SP1311, the presence of pSJ142 (SP1317) conferred about 3000-fold protection whereas no significant protection was detectable when ORF13 was present as a single copy within Tn*5252* as part of the chromosome in strain SP1402. When a 0.5 kb *Xba*I

fragment internal to ORF13 was deleted from within pSJ142 (SP1324), the UV survival disappeared indicating a definitive role for ORF13 in the observed protection. SP1323 and SP1405, lacking the whole UV-resistance determinant and part of ORF13, respectively, were as sensitive to UV-irradiation as SP1311. Fig. 12 shows the UV-killing curves of the pneumococcal strains used in this study.

Complementation Studies in *E. coli*

In order to investigate the possibility that the UV-protection determinant from Tn*5252* would be able to complement a *umuC* mutation in the chromosome of *E. coli,* the plasmid, pSJ142, was introduced into *E. coli* RM1140 (*umuC36*). The survival rate of cells containing pSJ142 increased about 100-fold in comparison of RM1140 cells (Fig. 13). Interestingly, the survival rate of RM1140 cells containing the recombinant plasmid was slightly higher than of AB1157 cells (*umuCD*$^+$) (Table 8). Also, the level of survival of RM1140(pSJ142) was very similar to that of RM1140(pSE117) and RM1140(pKM101). Plasmids, pSE117 (54) and pKM101 (56), carry *umuDC* and *mucAB* operons, respectively. As expected, when the vector plasmid, pAT29, was introduced into RM1140 no increase in the survival rate was observed (Fig.13). The results suggested that the operon from Tn*5252* played similar functions as those of the *umuDC* and *mucAB* operons from other systems and was able to complement these functions in *E. coli.*

Figure 12. Effects of UV irradiation on survival of *S. pneumoniae* strains: -□-, SP1311; -■-, SP1317; -O-, SP1402; -●-, SP1405; -Δ-, SP1323; -◇-, SP1324. The numbers represent the averages obtained from at least two independent experiments.

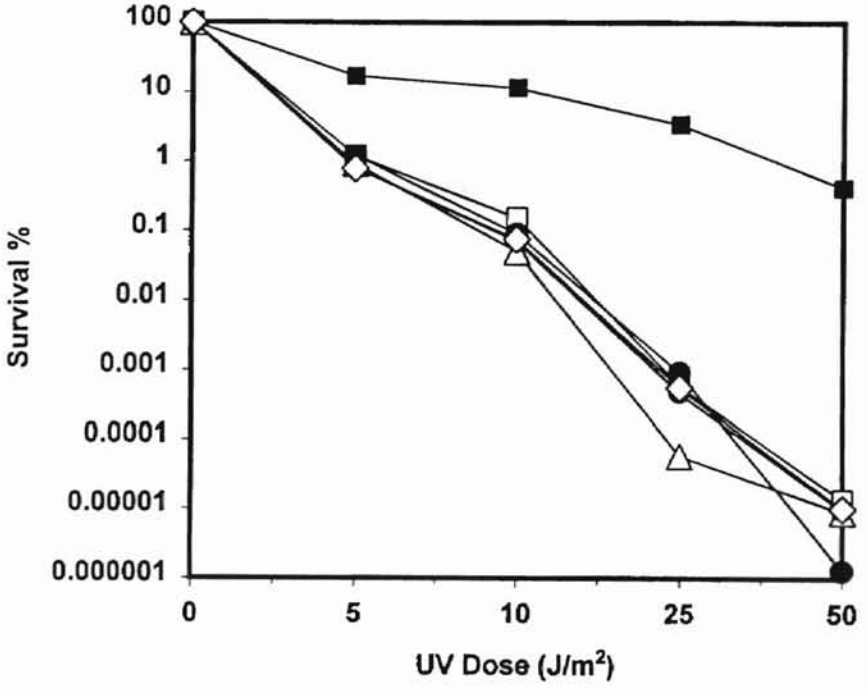
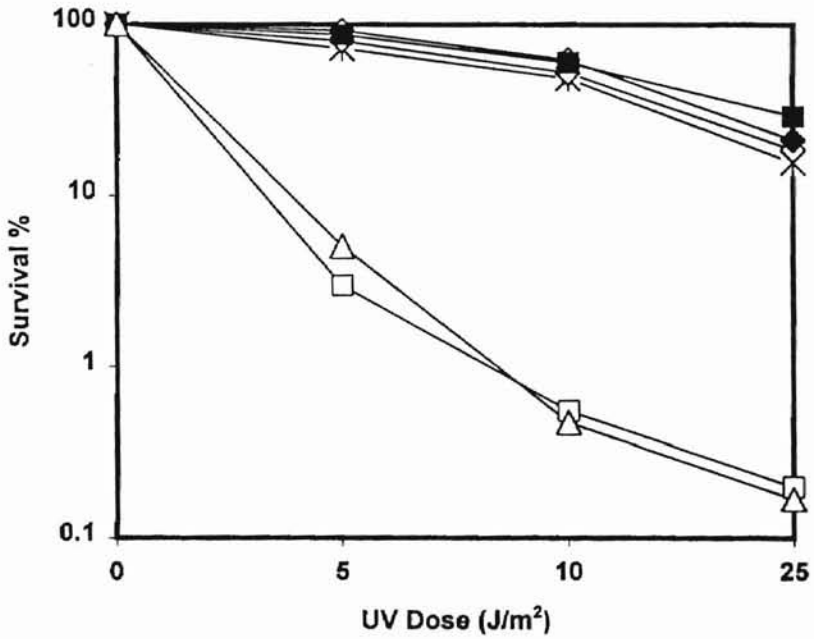

100
10
1
0.1
0.01
0.001
0.0001
0.00001
0.000001
Survival %
0
5
10
25
50
UV Dose (J/m²)

Figure 13. Effect of UV irradiation on survival of *E. coli*: -✳-, AB1157; -□-, RM1140; -■-, RM1140(pSJ142); -Δ-, RM1140(pAT29); -◇-, RM1140(pSE117); -◆-, RM1140(pKM101). The numbers represent the averages obtained from at least two independent experiments.


100
10
1
0.1
Survival %
0
5
10
25
UV Dose (J/m²)

SOS Mutagenic Response

Although pneumococci are capable of dark repair of UV-damaged DNA (27, 55), a distinct lack of the error-prone repair function of the SOS system has been documented (27). To determine whether the operon containing ORF13 conferring UV resistance was due to error-prone polymerization of DNA, pneumococcal cells carrying pSJ142 (SP1317) were exposed to various doses of UV and screened for the appearance of mutations in various genetic loci. The results are given in Table 7. For both markers, optochin and fusidic acid resistance, significant number of mutants appeared after UV treatment as compared to SP1311 cells. For reasons not clear, the optochin resistant mutants obtained were 20-fold higher than those of the fusidic acid resistance.

To determine whether the UV-protection operon of Tn*5252* could complement the SOS mutagenic function in *E. coli*, cells carrying pSJ142 were screened for *his*$^+$ revertants following various levels of UV treatment on minimal medium plates lacking histidine. *E. coli* AB1157 yielded substantial number of mutants as compared to RM1140 carrying the *umuC36* mutation. As expected, SOS mutagenic function was restored in RM1140 upon the introduction of multicopy plasmids carrying either the native *umuDC* operon in pSE117 or the mucAB operon in pKM101. Interestingly, the level of UV mutagenesis was highest with RM1140 cells carrying pSJ142 whereas no revertants were scored among the cells carrying the vector plasmid (Table 8)

Table 7

Effect of the UV-Protection Operon of Tn*5252* on UV-Induced Mutagenesis of Pneumococcal Strains

Strain	UV dose (J/m^2)	Survival %	Mutants per 10^8 survivors
S.pneumoniae			**Fus^r**
SP1311	0	100	0
	10	0.152	50
	25	0.00059	40
SP1317	0	100	20
	10	11.56	310
	25	3.4	6220
			Opt^r
SP1311	0	100	0
	10	0.152	0
	25	0.00059	40
SP1317	0	100	30
	10	11.56	4420
	25	3.4	113000

Fus^r, resistance to fusidic acid; Opt^r, resistance to optochin

Table 8

Effect of the UV-Protection Operon of Tn*5252* on UV-Induced Mutagenesis of *E. coli* Strains

Strain	UVdose (J/m^2)	% Survival	Mutants per 10^8 survivors
E. coli			**His^+**
AB1157	0	100	0
	5	72	18
	10	48	52
	25	15.5	189
RM1140	0	100	0
	5	3	0
	10	0.55	0
	25	0.2	0
RM1140(pSE117)	0	100	0
	5	80	40
	10	52	187
	25	18.5	310
RM1140(pKM101)	0	100	0
	5	92	302
	10	62	706
	25	21	880
RM1140(pSJ142)	0	100	0
	5	87	160
	10	60	264
	25	29	950
RM1140(pAT29)	0	100	0
	5	5.1	0
	10	0.47	0
	25	1.17	0

His^+, histidine prototrophic revertants

These experiments proved that the UV resistance operon from Tn*5252* confers protection against UV-irradiation, and probably against other DNA damaging agents, by increasing the number of induced mutations in the host. The results also suggested that this operon works the same way as *umuDC* and *mucAB* operons from other systems, including *E. coli.*

The Role of ORF13 in the Conjugal Transfer of Tn*5252*

To assess the relevance of ORF13 in the conjugal transfers of Tn*5252*, SP1400 cells, carrying a deletion mutation in ORF13 in a nov^r background, were used as donors in filter-mating experiments with *S. pneumoniae* as well as *S. pyogenes* recipient cells.

Three different *S. pneumoniae str*^{r} strains were used as recipients: SP1308, SP1311, SP1314. The construction of these strains has been previously described. The *S. pyogenes* strain used in this study as recipient is ATCC19651(Opt^r). SP1265, a *nov*^{r} strain carrying Tn*5252* with pVA891 inserted at a locus that is not involved in transfer, was used as control donor in the conjugation experiments.

After mating, the transconjugants were selected on CAT agar containing streptomycin and erythromycin when *S. pneumoniae* strains were used as recipients. When *S. pyogenes* was used as recipient, transconjugants were selected on CAT agar containing optochin and erythromycin. The Em^r

transconjugants were then replica-plated for unselected markers (transposon marker and donor marker). The results are shown in Tables 9 and 10. Comparison of the frequency of the transfer of SP1265 and SP1400 indicated that the transfer of the transposon was not affected by ORF13 deletion mutation. In fact, a slight increase in the transfer frequency was observed when the ORF13 mutant was used in the conjugation experiment with *S. pyogenes* as recipient.

Table 9

Effect of the ORF13 Mutation on the Conjugal Transfer Properties of Tn*5252* to *S. pneumoniae*

Donor	Recipient	Selection	Transconjugants per Donor
SP1265	SP1308	Em, *str*	5.8×10^{-6}
SP1400	SP1308	Em, *str*	1.0×10^{-5}
SP1265	SP1311	Em, *str*	1.6×10^{-7}
SP1400	SP1311	Em, *str*	2.0×10^{-7}
SP1265	SP1314	Em, *str*	8.6×10^{-7}
SP1400	SP1314	Em, *str*	1.4×10^{-7}

The results are the average from two independent matings.
SP1265 is *nov*^{r} and carries Tn*5252* with pVA891 inserted at a locus that is not involved in transfer.
SP1400 is *nov*^{r} and carries Tn*5252* with pVA891 inserted within ORF13.
SP1308, SP1311 and SP1314 are *str*^{r} pneumococcal strains

Table 10

Effect of the ORF13 Mutation on the Conjugal Transfer Properties of Tn*5252* to *S. pyogenes*

Donor	Recipient	Selection	Transconjugants per Donor
SP1265	*S.pyogenes*	Em, *opt*	1.2×10^{-6}
SP1400	*S.pyogenes*	Em, *opt*	4.3×10^{-5}

The results are the average from two independent matings.
SP1265 is *nov*$^{-}$ and carries Tn*5252* with pVA891 inserted at a locus that is not involved in transfer.
SP1400 is *nov*$^{-}$ and carries Tn*5252* with pVA891 inserted within ORF13.
S. pyogenes ATCC 19615 (*str*^{r}) was used as donor.

CHAPTER VI

CONCLUSIONS

We have determined the nucleotide sequence of a 3.3 Kb DNA at the right end of the DNA relaxase gene, the product of which is thought to be involved in the site-specific nicking of the circularized transposon molecule prior to its conjugal transfer. The sequence data revealed the presence of an operon-like region containing a consensus promoter and *rho*-independent transcription terminator-like sequences. Four ORFs were found, two of which were significantly similar to the proteins involved in error-prone repair of UV-damaged DNA.

Homology alignment of ORF13 protein in Tn*5252* with UmuC homologs from other systems indicated that, while the proteins originating from Gram-negative bacteria were more similar to each other, those from Gram-positive sources formed a distinct group indicating the evolutionary divergence of the two types. Plasmids carrying this segment of the transposon DNA were able to confer UV-induced mutagenic response and survival following exposure to UV in Gram-positive as well as Gram-negative bacteria devoid of error-prone repair capability.

Deletion of a 0.5 kb DNA from within ORF13 led to the abolition of the observed SOS mutagenic response demonstrating the involvement of this region of DNA in the repair of UV-damaged genetic material by introduction of mutations. This is the first demonstration of SOS induced mutagenic response in pneumococcus.

Two ORFs, encoding proteins of about 46 kDa and 15 kDa, have been noted in the most studied *umu* and *muc* operons of Gram-negative bacteria (25, 80) whereas four ORFs are present within the SOS operon of Tn*5252*. The role of the products of ORF11 and ORF12, if any in SOS mutagenesis, remains unknown at present. The *imp* operon of the IncI plasmid, TP110 (52), also has been shown to carry an ORF capable of encoding a 9.5 kDA protein with unknown function in addition to the UmuD and UmuC homologs. Further studies should establish the role of these proteins in the SOS response in pneumococcus.

According to the currently held model for SOS response, drawn mostly from studies in *E. coli*, the activated RecA* protein stimulates the autocleavage of LexA as well as the UmuD to UmuD'. The UmuC$(D')_2$ complex, by a mechanism not currently understood, enables DNA polymerase to continue trough the DNA lesions in an error prone manner (80). The finding that the SOS-related gene products of Tn*5252* could be processed in such a way to restore the UV-induced mutability in *E. coli* demonstrates that the structural and mechanistic details of this class of proteins are probably conserved in a wide range of bacterial species. It was also intriguing to find that the highest similarity to the

ORF14 protein, the homolog of UmuD, was the repressor of the fructosyltransferase gene of *S. mutans* which carries the conserved residues found in the LexA family of proteins and has been implicated as a virulent factor in the development of dental caries (75).

Surprisingly, plasmids are rare in clinical isolates of *S. pneumoniae* even though they have the capacity to receive and maintain plasmids from other streptococci via transformation under laboratory conditions. Recent emergence and dissemination of multiple antibiotic resistance in pneumococci has been chiefly due to the conjugative transposons (14). These novel elements seem to have functionally replaced plasmids in this species. The chloramphenicol resistance determinant in Tn*5252* has been shown to be flanked by direct repeats of an IS-like element which very often leads to spontaneous "curing" (62). The remaining cryptic element is transfer-proficient (5, 62). The stability of Tn*5252*-like elements to persist under natural conditions even in the absence of antibiotic selection is probably due to other genes, not related to the conjugal transfer, which may enhance the survival of their hosts. The presence of genes involved in the SOS response in Tn*5252* is an example supporting this notion.

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