The Analysis of Plasmid Rearrangements Observed in the Soil Bacterium OR168 After the Introduction of Transposon Tn*5*

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ABSTRACT

Transposon Tn*5* mutagenesis has been used extensively in *Escherichia coli* and various other Gram-negative bacteria to produce both random and site directed mutants. The popularity of Tn*5* as a mutagen stems from its apparent random insertion into the genome, leading to non-leaky polar mutations. It also confers on many bacteria resistance to aminoglycosides, providing a strong selectable marker. The site of insertion can be mapped by Southern DNA hybridisation against a specific Tn*5* probe.

Tn*5*-containing derivatives of the Rhizobia-like soil isolate, OR168, were produced using the broad host-range suicide plasmid vector pSUP1011. After the transfer of pSUP1011 to OR168 via heterogeneric bacterial conjugation, stable OR168::Tn*5* exconjugants were selectively isolated at frequencies of approximately 10^{-4} per recipient. None of the 53 OR168::Tn*5* exconjugants screened showed the parental plasmid profile. Visible alterations to the plasmid profile were common with respect to the native plasmid profile. These events generally showed large deletions from, or additions to, the native replicons of OR168. The alterations also included a low incidence of a decrease in plasmid number. Analysis of the exconjugant population shows that the insertion of Tn*5* into the genome of OR168 may not be strictly random. It was shown that 66% of OR168::Tn*5* exconjugants screened contain a plasmid-borne Tn*5* element, with 90% of those involving Tn*5* insertion in the same episome. There is evidence that events other than classical conservative transposition have occurred after the introduction of pSUP1011 into the OR168 genome.

Screening of the isolated OR168::Tn*5* population for pSUP1011 vector sequences revealed the presence of the pSUP1011-derived RP4-*mob* fragment in 33 of 35 OR168::Tn*5* exconjugants containing a plasmid-borne Tn*5* element. Analysis also revealed the acquisition of Tn*5* alone, presumably by conservative transposition, occurred only twice in the 35 events involving a plasmid target. This suggests that another site within the RP4 fragment can act as a surrogate transposase recognition site. Alternatively, the insertion of the RP4-*mob*::Tn*5* sequence into a plasmid target may involve a site specific recombination process peculiar to the OR168 isolate.

No mechanism was elucidated for the formation of many of the alterations in plasmid mobility. Restriction fragment lengths in the immediate vicinity of the anomalous RP4-*mob*::Tn*5* insertion are identical in different plasmids. This may indicate sequence duplication among the OR168 plasmids. Such duplication may precipitate, through homologous recombination processes, the plasmid instability observed.

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ABBREVIATIONS

AMPPD	3-(2′-Spiroadamantane)-4-methoxy-4-(3″-phosphoryloxy) -phenyl-1,2-dioxetane
AMP~D	Unstable dephosphorylated intermediate of AMPPD degradation
AP	Alkaline phosphatase
Ap	Ampicillin
ATP	Adenine triphosphate
BCIP	5-Bromo-4-chloro-2-indolylphosphate
bp	Base pair
CCC	Covalently closed circular
Cm	Chloramphenicol
CsCl	Cesium chloride
CTAB	Hexadecyltrimethyl ammonium bromide
DIG	Digoxigenin
DM	Distance migrated
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded DNA
EDTA	(Ethylenedinitrilo) tetra-acetic acid
EtBr	Ethidium bromide
IE	Inside end (of IS*50* and Tn*5*)
IncF	Incompatibility group F plasmid
IncP	Incompatibility group P plasmid
IS	Insertion sequence
kb	Kilobase pairs
Km	Kanamycin
LB	Luria-Bertani medium
NBT	4-Nitro blue tetrazolium chloride
Nm	Neomycin
nt	Nucleotide
OC	Open circular
OE	Outside end (of IS*50* and Tn*5*)
oriT	Origin of transfer
RM	Relative electrophoretic mobility
Sp	Spectinomycin
SSC	Standard sodium citrate
ssDNA	single-stranded DNA
TBE	Tris-borate-EDTA
Tc	Tetracycline
TE	Tris-EDTA buffer
tra	Transfer genes
Tris	2-Amino-2-(hydroxymethyl)- 1,3-propanediol acetate
Tn	Transposon
TY	Tryptone Yeast extract medium

1. INTRODUCTION

1.1 ALTERATION IN GENOME ORGANISATION AND GENE TRANSFER IN BACTERIA

In bacteria there are a number of characterised processes that can lead to the rearrangement of segments of their genome. They involve both endogenous processes, which result in intragenomic rearrangements; and exogenous processes, which are precipitated by intercellular genetic exchange. The endogenous processes of genomic rearrangement include deletions, duplications, inversions, recombination and translocations of resident DNA sequences to new sites within the same genome. The exogenous processes include the three mechanisms by which genes can be transferred to new hosts: namely conjugation, transduction and transformation. In an evolutionary context, it is generally believed that such rearrangements, both endogenous and exogenous, are an important source of biological variability.

The movement and accommodation of mobile genetic elements (plasmids, viruses and transposons) facilitate rearrangement by introducing novel DNA sequences into the genome. The genetic mechanisms necessary for their movement are generally element-encoded and element-specific, although host functions may be involved with some aspects of the control and regulation of these mechanisms. The transfer of novel DNA sequences and their insertion into the new genome can involve the concerted action of two different mobile genetic elements. For example, the intergeneric movement of transposons is facilitated by their insertion into either conjugative-competent plasmids or temperate phages. In concert, the mechanisms allow for horizontal gene transfer, which are important and intrinsic procedures responsible for species diversity and microevolution (Mazodier & Davies, 1991).

Generally, the bacterial genome cannot be considered a fixed entity but has the capacity to undergo change induced by both endogenous and exogenous processes. The resultant changes can be manifested as alterations in both genome organisation and phenotype. Therefore, the structural organisation of the genome can be regarded as being quite dynamic, with rearrangement events being common and not necessarily deleterious.

The following sections of the introduction detail aspects of both endogenous and exogenous processes that help explain the genetic events observed in this study.

1.2 GENETIC RECOMBINATION

Genetic recombination is a ubiquitous phenomenon found in both prokaryotic and eucaryotic organisms and involves the exchange of genetic material between distinct DNA molecules.

Numerous studies have been carried out to help define the mechanisms and processes of genetic exchange and recombination, and to help explain the resulting genomic rearrangements. These studies have resulted in the development of three different broad-based models to describe and classify the types of recombination events seen. In accordance with terminology used by Low and Porter (1978), the three basic modes of recombination are (i) general, which takes place between two homologous DNA sequences; (ii) site-specific (both double and single), which involves a specific location; and (iii) illegitimate recombination which occurs without apparent regard to special sites or extensive sequence homology. These modes of recombination are briefly outlined below.

The mechanisms which result in simple recombination between DNA molecules can lead to events that have been well documented. These events include sequence deletions and duplications, gene amplifications, insertion of foreign DNA molecules, and the translocation of DNA sequences to new sites. Berg (1990), Shapiro (1985) and Terzaghi & O'Hara (1990) present recent reviews of current ideas of genetic recombination and how the mechanisms influence organisation of DNA at the local and global level within the genome.

The study of the phenomenon of genetic recombination, particularly events involving exchange between nonhomologous regions, has led to the realisation that genomes are not fixed, but are in constant flux. These studies have also revealed a remarkable similarity in the mechanisms responsible for recombination between diverse organisms.

1.2.1 General Recombination

The mechanics of the steps involved in general recombination basically involves alignment of homologous DNA strands, followed by strand exchange at the site of a nick or gap in the DNA. This exchange results in the formation of a heteroduplex joint, which involves DNA synthesis and strand cleavage (Holliday, 1964; Radding, 1982). This is followed by branch migration and ultimately, resolution and segregation. The initial strand exchange event forms a limited region of

heteroduplex DNA. Branch migration then enlarges the initial heteroduplex (Warner *et al.*, 1979). A second strand exchange and ligation between the opposite parent strand completes the crossover structure. Resolution of the crossover structure into recombinant molecules involves symmetric nicking at the position of the crossover junction. General recombination probably occurs in all prokaryotes. It is considered to be an important factor in formation of species-specific genetic diversity and in the repair of damaged DNA sequences.

General recombination requires extensive sequence homology between the DNA molecules undergoing recombination and depends on system-specific enzymes. In *Escherichia coli* general recombination is dependent on *recA* system genes. Genes analogous to *recA* are found in numerous bacteria, including *Rhizobium* species (Better & Helinski, 1983). The RecA protein plays a central role in general recombination, by promoting homologous pairing and strand exchange between DNA molecules in an ATP-dependent reaction (Cunningham *et al.*, 1980; West *et al.*, 1981). General RecA-dependent recombination is most efficient when it operates on long stretches (30 to 150 bp) of highly homologous (≥97%) DNA, although it can occur between DNA molecules with homologies as short as 20 bp or with a significant amount of heterology, but at a much lower frequency (Shen & Huang, 1986; Watt *et al.*, 1986).

The RecBCD peptides are also required for efficient general recombination in *E. coli*. The *recBCD* genes encode exonuclease V, which has a number of enzymatic activities. It acts as an ATP-dependent exonuclease on ss and dsDNA, as well as an endonuclease on ssDNA (Amundsen *et al.*, 1986). It also unwinds DNA and generates ss loops (Taylor & Smith, 1985). Evidence suggests RecBCD is initially required for strand unwinding and nicking during the general recombination process, which provides a substrate for the RecA strand transfer reaction. Later, RecBCD is required again for the resolution of crossover structures.

General recombination can result from a single reciprocal cross-over between repeat sequences present within the same molecule, or alternatively, repeat sequences in different molecules. As such events introduce an instability in the organisation of the genome, the occurrence of repeat sequences has to be limited to tolerable levels. Additionally, not all recombination products may be recovered from single reciprocal crossover events due to their nonviability.

1.2.2 Site Specific Recombination

Site specific recombination typically involves DNA molecules sharing little DNA sequence homology (within which strand exchange occurs). This mode of recombination is independent of RecA function (Fishel *et al,* 1981), but does require system-specific proteins for the break/join reactions which underlie strand exchange (Weisberg *et al,* 1983; and Hsu & Landy, 1984). Two types of site-specific recombination mechanisms have been characterised. These have been termed 'double site-specific' and 'single site-specific' recombination.

Single site-specific events by definition, require only a single specific DNA sequence on one of the participating DNA molecules (Sherratt *et al*, 1984). This site is intimately involved with the break/join reactions of DNA required for recombination. System-specific proteins essential for their recombination bind directly at this site. Examples of single site-specific recombination events include the insertion of transposable elements (see Section 1.5) and the integration of bacteriophage Mu (Pato, 1989).

Double site-specific events require highly preferred sites on both of the participating DNA molecules. One well documented example of double site-specific recombination is the integration and excision of the bacteriophage lambda in the *E. coli* chromosome (Thompson & Landy, 1989). This genetic exchange is promoted by system-specific enzymes (the host-encoded protein, IHF; and the phage-encoded protein integrase) which catalyse the precise breakage and joining of DNA at the defined recombination sites: the attachment site *attP* on the phage chromosome and the attachment site *attB* on the bacterial chromosome.

1.2.3 Illegitimate Recombination

Illegitimate recombination includes those events not classified as general or site-specific recombination. It has been surmised by some (Anderson, 1987; Ehrlich, 1989; Franklin, 1971;) that illegitimate recombination may be the result of errors by enzymes which break and join DNA or errors during DNA synthesis. Such events usually involve the recombination of DNA sequences with little or no homology, and generally occur at a considerably lower frequency than either general or site-specific recombination. Illegitimate recombination is associated with genetic rearrangements that occur as a result of non-reciprocal deletions and duplications. Rearrangements can also occur by a reciprocal mechanism resulting in inversions and intermolecular exchange at short direct repeats. As the evidence indicates that illegitimate

recombination can occur by several different mechanisms, only those with relevance to this study will be mentioned in this section.

Events such as transposase-independent precise and imprecise excision of transposon Tn*5* are a consequence of replication error, with the deletion endpoints frequently coinciding with short direct repeats (Collins *et al.*, 1982). The frequency of transposon excision is markedly reduced by shortening the terminal inverted repeats, or conversion of the long inverted repeats to direct repeats at the transposon ends (Berg *et al.*, 1983; Egner & Berg, 1981).

Deletions involve the creation of a single novel joint between two previously distant genetic elements, usually involving sites with microhomology. Collins *et al.* (1982) and Hagan & Warren (1983) reported the instability of inverted palindromes generated *in vitro* by deleting the central region of transposon Tn*5*. A slipped mispairing model has been devised to explain deletion formation involving both direct and inverted repeats (Weston-Hafer & Berg, 1989).

Similarly, direct repeats and tandem duplications, involving *recA*-independent mechanisms, are created by the formation of a single novel joint between regions containing short repeats (Edlund & Normark, 1981).

1.3 MECHANISMS FOR INTERGENERIC GENE TRANSFER

Many incidences of genomic rearrangement are the result of gene transfer events. The advent of molecular biology has revealed the widespread incidence of genetic exchange between large numbers of unrelated microorganisms. In nature, the mechanisms which enable the dissemination of genetic information include bacterial conjugation, transduction by bacteriophage, and natural transformation events. A recent review by Mazodier and Davies (1991) details intergeneric transfer in bacteria.

The transfer of genes between bacteria, in turn, has an impact on the evolutionary process which drives species diversification. An understanding or at least an appreciation of events seen in laboratory systems may lead to a better understanding of similar events in nature. The molecular biology of the prokaryotic gene transfer mechanisms important for understanding the procedures and observations of this study are summarised in the following sections.

1.3.1 Bacterial Conjugation

This gene transfer process is very widespread, with conjugation systems having been found in both Gram-positive and Gram-negative eubacteria, as well as archaebacteria (Rosenshine *et al.*, 1989). It involves the direct transfer of plasmid DNA between closely associated cells. This intimate association means that the DNA is protected from degradation by extracellular nucleases or heavy metals. It appears conjugation is the major mechanism for horizontal genetic exchange.

The basic sequence of events for conjugative plasmid transfer in Gram-negative bacteria has been obtained from extensive studies of incompatibility group F (IncF) plasmids (Willetts & Skurray, 1987; Willetts & Maule, 1985) and IncP plasmids (Guiney & Lanka, 1989; Wilkins & Lanka, 1992). The plasmids from both incompatibility groups appear to be mobilised by similar mechanisms although their respective transfer genes are organised differently. The genetic organisation, function and control of essential transfer (*tra*) genes of the plasmids in these incompatibility groups have still not been totally clarified.

Generally, conjugation requires a number of element-encoded and element-specific functions. These include the *cis*-acting origin of transfer (*oriT*) site and a number of *trans*-acting genes necessary for mating pair formation, and for the initiation, continuation and termination of DNA transfer, as well as for the control of these processes. Some of the products encoded by transfer genes can be utilised by plasmids of other incompatibility groups; while others are plasmid-specific.

1.3.1.1 Conjugation of IncPα Plasmids

For the self-transmissible IncP group plasmids, plasmid transfer initially involves the establishment of a mating pair and signalling that transfer should begin. This involves the formation of a relaxosome at the *oriT* site. The relaxosome is a stable complex of supercoiled DNA and *tra*-gene encoded proteins. It is regarded as an intermediate in the initiation of conjugative transfer of plasmid DNA (Nordheim *et al.*, 1980).

Following initiation, plasmid transfer involves a number of steps. Single-stranded and site-specific cleavage occurs within *oriT* (*nic* site) after formation of the relaxosome. The resultant nicked plasmid species appears to exist in a thermodynamic equilibrium with the covalently closed species (Pansegrau *et al.*, 1990). Subsequent strand displacement via rolling circle replication results in the transfer of a single stranded DNA molecule into the recipient cell in a 5′ to 3′ direction. The final steps involve ligation of the transferred strand at the *nic* site and

subsequent complementary strand formation in both donor and recipient cells to restore the duplex plasmid molecule.

The transfer genes of the IncPα group plasmids have a complex organisation. They are located in two regions separated by 10 kb and are designated the TRA1 and TRA2 regions. The TRA1 region consists of approximately 15 kb DNA and contains the *oriT* site. The TRA1 encodes at least 15 putative genes arranged in four transcriptional units (Fürste *et al.*, 1989; Waters *et al.*, 1992). The gene products of *traF, traG, traH, traI, traJ,* and *traK* are essential for plasmid transfer between *E. coli* cells. TraF and TraG proteins may be associated with the formation of mating pair complex, with TraF involved in pilus biosynthesis (Waters *et al.*, 1992). TraI and TraJ are involved in relaxosome formation which is stabilised by protein-protein interactions of the TraH polypeptide. The *oriT* is nicked by the TraI protein which remains bound to the ss 5′ end of the nicked strand and may facilitate the translocation of DNA from the donor to the recipient (Pansegrau *et al.*, 1990a; Pansegrau *et al.*, 1990b). TraK protein binds specifically to a number of small DNA sequences along the leading strand in the *oriT* region of the relaxosome and has the ability to compact DNA (Ziegelin *et al.*, 1992).

The TRA2 region [formerly Tra2/Tra3 (Pansegrau & Lanka, 1987)] consists of approximately 11 kb of DNA and contains loci for *kilB*, entry exclusion and phage sensitivity. The *kilBI* gene is essential for conjugative transfer although its function is not completely known. It is known that expression of *kilB* is lethal to the *E. coli* host without regulation by the appropriate *kor* determinants (Figurski *et al.*, 1982). It appears to be co-regulated along with the neighbouring *trfA* operon (Motallebi-Veshareh *et al.*, 1992). The TrfA protein initiates vegetative replication at *oriV*. The KilBI protein has homology to VirBII of *Agrobacterium tumefaciens* Ti plasmid and could be associated with protein export functions.

1.3.2 Transduction

Transduction is a gene transfer process mediated by bacteriophages. Although the process has no direct bearing on this study, it is mentioned in recognition that it is an important process of genetic transfer and ultimately genome rearrangement. The potential for transduction has important ecological significance in the natural environment, even with the inherent narrow host range for bacteriophage. Their narrow host range is due to specific receptor sites used by the phage to adsorb to host cells. This means movement of genetic material by transduction is generally limited to closely related species. But because the DNA is packed as a phage particle, it may persist for long periods of time in the environment (Zeph *et al.*,

1988). Also, the lysogenic activity of temperate phages means there is the potential to transfer any part of the host genome to another host.

Transduction occurs as a result of the encapsulation of host DNA during the lytic stage of the phage life cycle. The resultant transducing phage is normal in its ability to infect new host cells, but defective in its ability to direct the missing genes essential for completing its normal life cycle.

1.3.3 Transformation

Transformation is the third type of mechanism involved in the transfer of genetic material between bacteria. Again, it has no direct bearing on this study and is mentioned to complete the comparison between the processes of genetic transfer. It involves five stages: appearance of DNA in the environment, development of competence in the recipient host, binding of DNA to the cell surface, uptake of DNA by the recipient and integration (and expression) of the DNA (Trevors *et al.*, 1987).

Competence is a specific physical state of bacterial cells that allows nucleic acids to bind to the cell surface and be taken up. Little is understood about the general mechanism of competence development in bacterial cells. The specific physiological or genetic properties leading to competence appears to be species-specific. For example, the Gram-negative *Acinetobacter* species is naturally competent for transformation at high frequencies for most of its life cycle, whereas with *Escherichia coli* competence can be induced by treatment with salts. It appears the transformation procedures carried out in the laboratory mimic the natural occurrence of transformation (Trevors *et al.*, 1987). The actual frequency of natural transformation is unknown with both environmental factors (including the survival of naked DNA against nuclease degradation or its irreversible adsorption to soil materials such as clay) and host factors (such as functional integration of the transformed DNA in the host) strongly influencing the probability and outcome of the event.

1.4 BACTERIAL PLASMIDS

Plasmids are found widely distributed in prokaryotic organisms with almost every species of bacteria carrying them (Helinski *et al.*, 1985). Plasmids are extrachromosomal genetic elements which encode functions for their own replication and maintenance. Generally they are double-stranded covalently closed circular DNA molecules, although linear molecules have been isolated from *Streptomyces* species (Hayakawa *et al.*, 1979). They can be divided into two general classes: conjugative and nonconjugative plasmids. The former class encode functions for their infectious transmission. Nearly all naturally-occurring plasmids are either self-transmissible or possess an incomplete mobilization system. The conjugation mechanism is complex and not well characterised in molecular terms (see Section 1.3.1 for more details).

Nonconjugative plasmids can be mobilized if they contain *oriT* sequences and are able to supply the specific proteins needed to recognise their own *oriT*. They can use the components of another conjugative system expressed by a different plasmid in the cell to manifest mobility. This is achieved by providing the specific transfer functions *in trans*.

Plasmids can make up a sizable portion of the total DNA present in a bacterial cell. Generally, plasmids smaller than 30 kb have high copy numbers with 10-40 copies per cell; while plasmids greater than 30 kb have low copy numbers with only 1-10 copies per cell. Low-copy number plasmids usually encode functions for active partitioning systems to ensure their stable inheritance. To some extent, plasmid size and copy number are linked. The consequence of more information encoding for extra functions, such as the resolution of duplicated interlinked molecules and their partitioning into daughter cells, means a reduction in copy number that is coupled with an increase in plasmid size. This probably has significance for survival of the replicon with regard to the dynamics of DNA replication and cell division cycle.

1.4.1 Incompatibility Group P Plasmids

A lot of interest has been focused on conjugation of the incompatibility group P plasmids (IncP plasmids) because of their extensive host range among Gram-negative bacteria. Utilization of this feature has made them useful tools for genetic manipulation of a wide variety of bacteria. They are able to be conjugatively transferred to phylogenetically distant cells (Thomas & Smith, 1987) including both gram-positive (Trieu-Cuot *et al.*, 1987; Williams *et al.*, 1990) and gram-negative organisms (Guiney & Lanka, 1989) as well as yeast (Heinemann & Sprague, 1989;

Sikorski *et al.*, 1990). The IncP plasmids can vegetatively replicate in diverse gram-negative bacteria, although the conjugative transfer functions of these plasmids have a greater host range than that for their vegetative replication. This makes them well suited for the development of cloning vector systems and transposon delivery systems useful for both *intra-* and *inter*-generic transfer of DNA (Ditta *et al.*, 1985; Simon *et al.*, 1983b).

1.4.2 Native Plasmids of Rhizobia

Bacteria of the genus *Rhizobium* have the capacity to interact symbiotically with the roots of leguminous plants, forming nitrogen-fixing nodules. Many *Rhizobium* strains contain a number of different plasmid species (Prakash & Atherly, 1986). The number within a strain can vary from one to six, with up to 25% of the total DNA of some strains contained within plasmids. Generally, the symbiotic functions are encoded on both the chromosome and plasmids, with many of the essential genes for nodule formation and nitrogen fixation frequently found clustered on the symbiotic (pSym) plasmids.

Plasmids well in excess of 150 kb have frequently been reported and identified in many different *Rhizobium* strains. The presence of large plasmids appears to be a characteristic common to *Rhizobium*, with some species carrying more than one large plasmid. In the case of *R. meliloti* 1021, two megaplasmids in excess of 1.3 Mb have been reported. Both symbiotic and catabolic genes are scattered between these megaplasmids and the chromosome of this strain (Honeycutt *et al.*, 1993).

Another characteristic common to *Rhizobium* plasmids is the presence of reiterated sequences at levels unusually high for prokaryotic organisms. The scattering of reiterated sequences between different replicons has consequences on the internal plasticity of their genome. Plasmid rearrangements as a result of recombination between reiterated sequences has been shown using a 390 kb pSym plasmid construct in *R. phaseoli* CFN42 (Romero *et al.*, 1991). Recombination between the reiterated nitrogenase structural genes within the pSym contributed to the formation of both amplification and deletion products.

1.5 TRANSPOSONS

There are three types of transposable genetic elements: insertion sequences, retroelements and transposons. Transposable elements are discrete DNA sequences with the intrinsic ability to change their genomic location. They are diverse in structure and have been found in virtually all groups of organisms studied. Collectively, there are two essential features common to all transposable elements: they are delineated by end sequences that are required *in cis* for transposition, and they encode functions that control and facilitate that transposition.

Insertion sequences are the simplest type of transposable element. They only encode information for their own transposition. They have been found in both prokaryotes and eukaryotes, and are generally 700 to 2500 bp in length.

Retroelements are DNA or RNA sequences that encode for reverse transcriptase and/or integrase activity. There are a number of different classes of retroelements of which all but the structurally most simple (retrons) can transpose by the process of retroposition. Retroelements also include the retroviruses and because they encode for virion particles they are capable of moving to other cells (Temin, 1989). Host cell sequences can recombine with the retroviral sequence and be subsequently transposed to new sites in the host genome or be introduced into new hosts. This provides a means by which genome organisation is altered.

Transposons exist mostly as families of dispersed repetitive sequences in the genome. They are generally 2.5 to 7.0 kb long and are distinguishable from insertion sequences by carrying genes encoding functions other than those related to their transposition.

1.5.1 Transposon Tn*5*

The prokaryotic transposable element Tn*5* is a 5.8 kb segment of bacterial DNA (Berg, 1989) (see Figure 1.5.1 for a schematic of Tn*5* structure). It is classified as a composite transposon consisting of two almost identical terminal 1533 bp insertion elements, IS*50*L and IS*50*R, in inverted orientation flanking a 2754 bp central region. The central region consists of three co-transcribed antibiotic resistance genes but plays no direct role in transposition. The resistance genes of Tn*5* are the aminoglycoside 3′ phosphotransferase gene, *kan* (which confers resistance against kanamycin and neomycin), and genes encoding resistance against bleomycin and streptomycin (Mazodier *et al.*, 1985). The gene encoding streptomycin resistance is

Figure 1.5.1: Structure of Tn*5*.

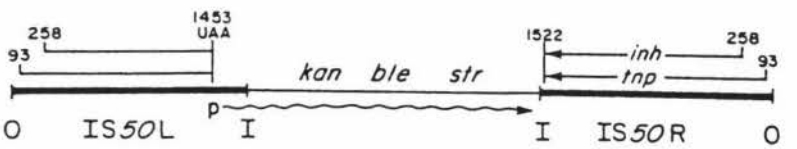


Figure 1.5.1a. Schematic showing organisation of Tn*5*. Solid lines: the 1533-bp insertion sequences IS*50*L and IS*50*R, present as terminal inverted repeats; transposon is indicated with the transposase (*tnp*) and inhibitor (*inh*) reading frames above the line and the antibiotic resistance transcript below it. The position of the outside (0) and inside (I) end sequences are indicated. The ochre protein fragments from IS*50*L are also shown. The nucleotide coordinates at which proteins begin and end are indicated. *kan*, *ble*, and *str* are the genes encoding resistance to kanamycin, bleomycin, and streptomycin respectively. From Berg *et al.*, 1988.

O end: $\overset{1}{C}TGACTCTTATACACAAG\overset{19}{T}AGCGT$

DnaA box: $TTAT^{C}_{A}CA^{C}_{A}A$

I end: $\overset{1}{C}TGTCTCTT\underline{GATCAGATC}\overset{19}{T}\underline{TGATC}$

Figure 1.5.1b. Outside and inside end sequences of IS*50*. Fine structure deletion mapping indicated only the first 19 bp of the OE and IE sequence are needed for efficient transposition of IS*50*. Large letters indicate positions where the IE and OE sequences are matched. The consensus binding sequence for DnaA protein is shown for the OE sequence. For the IE sequence Dam methylation sites are underlined. From Berg, 1989.

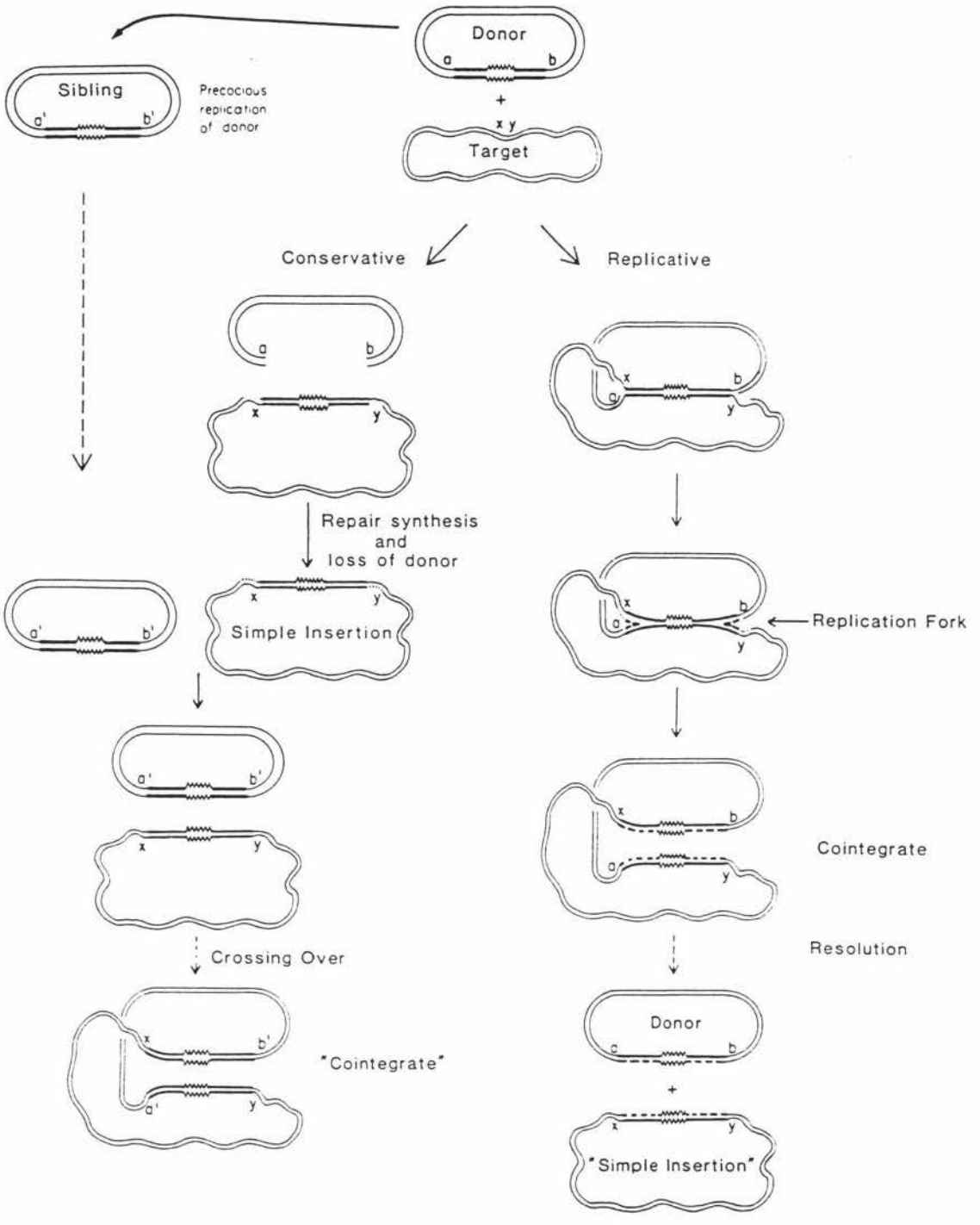


Figure 1.5.2: Models for transposition mechanisms.

Formation of cointegrates and simple insertions by both conservative and replicative transposition. Transposition is to the x-y target. The first step in conservative transposition involves excision of the element from the donor replicon (which is degraded) and insertion into a staggered cut in the target replicon. The single-stranded DNA at the insertion site is repaired to form a characteristic short direct repeat of target sequences bounding the insert. Precocious replication of the donor replicon may lead to duplicate copies of the transposon: one at the original site, and one at the new site. Apparent "cointegrates" could arise subsequently by a crossover between the transposed element in the target replicon and another element on a sibling of the donor replicon. The first step in replicative transposition involves single-stranded nicks at the element-vector junctions and a staggered cut in the target, followed by ligation of donor and target strands to form the 'Shapiro intermediate' structure. Replication of the element after joining of the element and target DNAs results in a cointegrate composed of the two parental replicons bounded by direct repeats of the element. Resolution is mediated by a site-specific resolvase in some elements or by host-mediated general recombination. An apparent "simple insertion" bounded by the characteristic short direct repeat of the target sequence could arise subsequently by a crossover (resolution) between daughter elements. Symbols: heavy line, transposable element; a and b, loci adjacent to the element in the donor; x and y, loci bracketing the target site; zig zag, central region of transposon; dotted line, repair synthesis; heavy dashed line, newly replicated strands. From Berg *et al.*, 1989.

usually cryptic in enteric bacteria, but is expressed in many non-enteric bacteria. For the *ble* gene product to confer resistance against bleomycin it requires the concomitant expression of the host genes *aidC* and *polA*. Blot *et al.* (1993) also found the Ble protein induces AidC expression, increasing the repair of spontaneous DNA lesions. Because of this activity, it has been suggested Tn*5* confers a selective advantage on the cell.

The two IS elements of Tn*5*, IS*50*L and IS*50*R, differ only in a single base pair (Rothstein & Reznikoff, 1981). IS*50*R contains transcripts for both the transposase gene, *tnp*, and the overlapping inhibitor of transposition gene, *inh*, which utilises a promoter downstream of the transposase promoter (Johnson *et al.*, 1982; Isberg *et al.*, 1982). The IS*50*L element contains an ochre mutation resulting in the production of two nonfunctional truncated proteins analogous to Tnp and Inh. The ochre mutation has also created a new constitutive promoter specific for the antibiotic resistance genes.

Both IS*50* elements of Tn*5* (see Figure 1.5.1) are delineated by two unique 19-bp *cis*-acting end sequences that share some sequence and function similarity. These ends are called outside ends (OE) and inside ends (IE) by virtue of their proximity to the central region (Johnson & Reznikoff, 1983; Sasakawa *et al.*, 1983). Both end sequences are essential for transposition, with the distinguishing feature between IS*50* and Tn*5* transposition being the choice of the transposable element end sequences used. Tn*5* transposition uses two OE sequences, whereas IS*50* transposition uses an OE and an IE sequence. Hence, the IS*50* element is capable of independent transposition.

1.5.2 Transposition in Prokaryotes

The RecA-independent recombination process frequently referred to as transposition, generally involves the insertion of a discrete DNA segment known as a transposable element into one of many alternative sites within the bacterial genome. Like all site-specific recombination events, transposition involves precise breaking of DNA in both donor and target molecules, followed by ligation between the transposon termini and the target molecule. Both events are highly regulated with transposition being mediated by DNA sequence-specific sites and transposon-encoded enzymes called transposase which act at the site of DNA cleavage.

Unlike site-specific recombination events, however (which result in precise cleavage and rejoining of all broken strands), transposition involves some DNA synthesis. This synthesis is specified by the DNA repair function of DNA polymerase I enzyme

and is responsible for the synthesis of the short direct repeat sequence found bordering the site of the insertion.

Another important difference between site-specific recombination and transposition is that during transposition the donor is not reconstructed to its wild-type pretransposon sequence, as is the case during site-specific excision/resolution events. This is the consequence of the short direct repeat sequences bounding the insertion site.

The transposable elements of Gram-negative organisms can be classified into three general classes based on their mechanism of transposition. The Tn*3*-like elements transpose through a two-step replicative mechanism (see Figure 1.5.2). This involves the formation of a branched structure, the 'Shapiro intermediate', between the donor and recipient replicons at the transposon site (Shapiro, 1979). Replication of the transposon within this intermediate results in the fusion of the two replicons and the formation of a 'cointegrate' structure. Resolution by site-specific or general recombination regenerates the donor and recipient replicons, each carrying a copy of the transposon.

Other transposons, such as Tn*10* and Tn*903*, transpose by a conservative mechanism (see Figure 1.5.2), generating only simple insertions with no net replication of the transposon. The ends of the remnant donor DNA strand are not rejoined, resulting in it being exonucleolytically degraded. The ability of conservative events to increase the number of Tn*5* elements in a genome may result from precocious replication of the replicon containing the element. A transposition event following precocious replication will not result in the net loss of the donor replicon. This event is partially dependent on factors which control DNA replication in the host bacteria, and occurs very infrequently.

Tn*9*, bacteriophage Mu and related viruses make up the third class of transposable elements, which can transpose using either the conservative or replicative mechanisms. For example, during lytic growth, Mu transposes replicatively. Successive rounds of cointegrate formation produces the 100 or so copies of the genome required for successful infection. As a result of this means of replication, the ends of Mu are always flanked by adjacent host DNA. By comparison, the lysogenic insertion of Mu is conservative transposition, with the loss of the adjacent host DNA.

1.5.2.1 Tn5 Transposition

The proposed mechanism of transposition for Tn*5* is conservative. In this mechanism, the Tn*5* is separated from its donor vector by precise double-stranded breaks at each outside end (OE) of the element. It inserts into a target DNA molecule at the site of a 9-nucleotide staggered cut.

The transposition of Tn*5* is mediated by the IS*50*R-encoded 476-amino-acid transposase (Tnp) acting preferentially *in cis*, on OE-OE sequences (or OE-IE sequences for IS*50* transposition) located close to the site of Tnp synthesis on the same replicon (Isberg *et al.*, 1982; Johnson *et al.*, 1982). This infers that one Tn*5* element (or IS*50*R sequence) rarely promotes transposition of a second element in the same cell. Gel retardation analysis shows Tnp binds specifically to the 19-bp OE sequence (de la Cruz *et al.*, 1993). The N-terminal 55 amino acids of Tnp may be required for this binding, even though Tnp contains no recognizable DNA-binding motif.

The 421-amino-acid inhibitor protein (Inh) is encoded in the same reading frame as Tnp, but from a different translational initiation site. Inh mRNA is also transcribed from its own promoter site, adjacent to the transposase promoter. This permits separate control of Tnp and Inh synthesis (Krebs & Reznikoff, 1986). Inh is known to inhibit transposase activity *in trans* (Isberg *et al.*, 1982; Yin & Reznikoff, 1988). Inh does not bind to the OE DNA, but enhances the OE binding of Tnp when it is added to reaction mixtures containing limiting amounts of Tnp. There is also evidence that Inh forms oligomers with Tnp (de la Cruz *et al.*, 1993). This suggests Inh does not competitively inhibit transposition of Tn*5* by blocking Tnp-DNA binding site(s), but rather suggests Inh binds with Tnp and that the resulting Tnp-Inh oligomers have altered DNA binding activity, which may inactivate the transposition function of Tnp.

1.5.2.2 Host Factors Influencing Tn5 Transposition

There are a number of host-encoded factors that are implicated in Tn*5* transposition. Some of these host factors suggest a link between transposition and chromosome replication and partition. There is no DNA synthesis during Tn*5* transposition other than to fill the gaps in the target DNA, resulting in the 9-bp direct repeats at the target site. The gap repair is carried out by the host encoded DNA polymerase I gene. Certain mutations in the *polA* gene result in a 20-fold decrease in the frequency of transposition events involving Tn*5*. *polA* mutants defective in both the polymerase and 5′ to 3′ exonuclease activities of DNA polymerase I, are deficient in transposition (Sasakawa *et al.*, 1981; Syvanen *et al.*, 1982).

Other host proteins also play critical roles in Tn*5* transposition. The Dam protein, DNA N^6-adenine methyltransferase, is involved in postreplicative methylation of adenine residues at two GATC sites overlapping the -10 region of the Tnp promoter (see Figure 1.5.1b). It appears *dam* methylation down-regulates the synthesis of the Tnp transcript and up-regulates the synthesis of the Inh transcript (Yin *et al.*, 1988). The 19-bp IE sequence also contains two *dam* methylation sites (see Figure 1.5.1b). Their methylation inhibits their use as transposition substrates. There is a third GATC methylation site at residues 21 to 24, in the 3′ direction, outside the IE sequence.

The three Dam sites also overlap a Fis binding site (Weinreich & Reznikoff, 1992). Fis (factor for inversion stimulation) is a small DNA-binding protein, with a variety of functions. The *E. coli* Fis protein functions in many diverse biological systems including recombination, transcription, and DNA replication (Finkel & Johnson, 1992). Its activity includes the ability to stimulate several site-specific DNA inversion reactions and the ability to enhance the chromosomal excision of Lambda. Although not essential for transposition, Fis is thought to indirectly influence the frequency of transposition events. Observations suggest that during exponential growth, high levels of Fis enhance transposition of both Tn*5* and IS*50*, while in *dam*$^-$ bacteria, IS*50* transposition is inhibited (Weinreich & Reznikoff, 1992).

Using mutational analysis of Tn*5* to study its transposition in *E. coli*, several host factors have also been implicated for the control and effect of its movement. It is thought DNA gyrase is required to supercoil target DNA in preparation for transposition. The gyrase protein may also be important for facilitating local melting and sequence recognition by the transposase protein (Isberg & Syvanen, 1982).

The DnaA protein, required for replication initiation of many chromosomal and plasmid replicons in bacteria, is also required for Tn*5* transposition (Yin & Reznikoff, 1987). DnaA binds cooperatively to a repeated 9-bp sequence at the chromosome origin, permitting the formation of the relaxosome complex. One copy of the consensus sequence for the DnaA binding site is also present at the OE of IS*50*. Along with methylation sensitivity, this suggests transposition is linked to active replication.

1.5.3 DNA Rearrangements Associated with Tn*5*

Composite transposons such as Tn*5* can cause, and are associated with many types of genetic rearrangement including deletions, inversions, and replicon fusions (cointegrate formation).

The existence of two or more copies of the same transposable element within the same genome creates circumstances that facilitate general recombination events. Tn*5* contains two copies of IS*50* in an inverted orientation which have the potential to independently transpose. The precocious replication of the donor replicon containing Tn*5*, followed by transposition of either Tn*5*, or its component IS element, results in an increase in the copy number of the element. Generalised recombination between the homologous sequences creates cointegrate structures due to replicon fusion (see Figure 1.5.2).

Conservative transposition involving cleavage of the IE sequences, rather than the OE sequences, by the transposase results in inverse transposition with the loss of the central core region of the transposon, which is replaced by the new target replicon (Nag *et al.*, 1985). The proposed products of intramolecular inverse conservative transposition also results in rearrangements. Depending on the relative orientations of the element and the target site during inverse transposition, the product may retain the two IS elements of the parental transposon, which are now separated by an inverted sequence, or it may consist of two smaller circular DNA molecules, each containing one IS element and part of the original replicon. Because of large deletions, the viability of the products will depend on the retention of vital functions such as an origin of replication (Berg *et al.*, 1989).

Precise excision of transposons also occurs, resulting in reversion of the insertional mutation along with the loss of the transposon plus one copy of the small direct repeat sequence (Egner & Berg, 1981). Excision of Tn*5* occurs at frequencies ranging from $<10^{-10}$ to 10^{-4}, depending on the exact Tn*5* insertion site. This process is unrelated to transposition: transposition requires the element-encoded transposase; while precise excision is thought to be controlled by mechanisms which control general recombination (see also Section 1.2.3 and Lundblad *et al.*, 1984).

The formation of deletions of host DNA adjacent to the outer end at the insertion site has also been reported (Tomcsanyi *et al.*, 1990). These transposition-associated adjacent deletions are thought to be the result of nonproductive attempts at transposition (Jilk *et al.*, 1993).

1.5.4 Tn*5* Mutagenesis and Plasmid Marking

Transposon Tn*5* mutagenesis is an important technique for creating insertion mutations. On its introduction into a cell lacking Tn*5*, the transpositional movement of Tn*5* is assisted by the absence of any of the Tn*5*-encoded *trans*-acting inhibition protein, Inh. This protein is thought to block transposition by binding to the *cis*-active Tnp and altering its DNA-binding activity. After the initial transposition in the new cell, further transposition is primarily prevented by the presence of Inh. Usually this creates insertion stability for the Tn*5* at its initial transpositional site. Secondary transposition occurs at very low frequencies.

Insertion of Tn*5* into a gene results in the complete dysfunction of that gene. The resulting mutant may be phenotypically scorable, making the technique useful for tagging genes of interest. Factors which make Tn*5* valuable as a mutagenic agent are those which are inherent features of Tn*5*. This includes the fact that unlike replicative transposons, Tn*5* is incapable of self-replication. It also inserts mostly at random into the genome, leading to non-leaky mutations. Meade *et al.*, (1982) reported spontaneous prototrophic reversion of Tn*5*-induced mutants occurring at rates between 10^{-8} and 10^{-10} in *R. meliloti*. They also reported the very low frequency of secondary transposition events in this organism suggesting the exconjugants isolated after Tn*5* mutagenesis are very stable.

Another important feature for using Tn*5* as a mutagen is the ease with which the mutants of interest can be selected and characterized. The insertion of Tn*5* leads to the acquisition of neomycin antibiotic resistance, which allows not only a primary screen for insertional events, among which can be found the mutations of interest, but also allows for the genetic and physical mapping of the site of the transposon's insertion.

Early studies of Tn*5* insertion suggested that it inserts randomly into many sites in any given gene. More detailed genetic mapping of Tn*5* insertion sites in the *E. coli* genome has revealed that the transposon inserts preferentially at certain target sequence hot spots. It was found that G/C pairs are present at each end of the 9 bp duplicated sequence at these hot spots (Berg *et al.*, 1983; Nag *et al.*,1985). This pattern of insertion may reflect the involvement of a number of host factors and topological features of the DNA in transposition. But the relatively relaxed insertion specificity and its ability to transpose in many different bacterial species has led to the wide use of Tn*5* as a selectable mutagenic agent in molecular genetics.

1.6 AIMS

There is considerable interest in understanding and characterising the plasmid diversity seen among bacteria of the genus *Rhizobium* (Hartmann & Amarger, 1990; Laguerre *et al.*, 1992; Rastogi *et al.*,1992). Historically, the phylogenetic classification of these Gram-negative soil bacteria has been based primarily on their ability to establish nitrogen-fixing symbioses with leguminous plants, with the relationship being host-specific (Jordon, 1984).

Since there is no selective means to isolate Rhizobia directly from soil, strains are commonly isolated and typed solely on the basis of the genus of its effective plant host. For instance, *R. leguminosarum* consists of three different biovars (bvs.), namely bv. *phaseoli*, which nodulates *Phaseolus vulgaris* (common bean); bv. *trifolii*, which nodulates *Trifolium* species (clover); and bv. *viciae*, which nodulates the cross inoculation group including *Lens* (lentils), *Pisum* (peas), and *Vicia* (beans). *R. meliloti* nodulates some species of *Medicago* (lucerne), *Melilotus*, and *Trigonella*.

Many genetic determinants of *Rhizobium*-legume interactions are encoded by genes carried on the large p*Sym* plasmids and other cryptic plasmids (Brom *et al.*, 1992; Hynes & McGregor, 1990). For many Rhizobia, some of these resident plasmids are mobilisable and can be transferred between different species of Rhizobia. This may lead to the conversion of specificity from one host to another (Johnston *et al.*, 1978; Broughton *et al.*, 1987). The intergeneric transfer of mobilisable plasmids can lead to alterations in plasmid profile through recombination between homologous or reiterated sequences. Alterations in plasmid profiles can also result from amplification, deletion or cointegration events (Brom *et al.*, 1991; Rastogi *et al.*, 1992; Romero *et al.*, 1991).

The incidence of plasmid-mediated genomic rearrangement has major implications for the ecological fitness and survival of the organism, especially in specialised habitats, where the genetic information encoded by the DNA involved in the rearrangements may provide some selective advantage.

As a historical perspective of events that initiated this study, work by O'Hara (1989) that involved following the fate of the Tn*5*-labelled p*Sym* plasmid of *Rhizobium leguminosarum* bv *trifolii*, revealed instances of extensive alterations to the plasmid profiles of recipient hosts after the intergeneric conjugative transfer of the Tn*5*-labelled p*Sym*. The genome of *R. leguminosarum* strain 2668 contains approximately 5800 kb of DNA, organised into two different types of replicons - the chromosome

and four very large extrachromosomal plasmids. These correspond in size to approximately 600 kb, 370 kb, 200 kb and 190 kb, making up approximately 25% of the genome.

Plasmid profiles, prepared using horizontal Eckhardt gel electrophoresis, show a stable band pattern for these four plasmids. Unpublished work by Terzaghi and Cleaver, carried out to elucidate this phenomenon of plasmid rearrangement, showed the plasmid pattern in the wild-type 2668 strain was unchanged over many generations of growth under laboratory conditions. This supports the idea of a stable plasmid population within the cell.

They also showed that the introduction of the pSUP1011 suicide vector bearing Tn*5* into the 2668 genome results in extensive rearrangement of the plasmid bands. The rearrangements were depicted as alterations in plasmid mobility using Eckhardt gel electrophoresis, and only occurred in the lower doublet bands corresponding to the 200 kb and 190 kb plasmids. The resulting rearrangements to the plasmid profile are varied, but the products appear to be stable. The variations seen are characterised by: (i) the complete loss of a particular band,
(ii) the appearance of additional bands, and
(iii) the alteration of band size with both increases and decreases in size.

These rearrangements appear to mimic those observed on a comparative analysis of plasmid profiles of indigenous field populations (Hartmann & Amarger, 1991; Hynes & O'Connell, 1990; Young & Wexler, 1988). Frequent genomic rearrangements (in the range of 10^{-2} to 10^{-3}) have been commonly observed in different Rhizobial groups, including *Bradyrhizobium japonicum*, *Rhizobium leguminosarum* bv. *phaseoli* and bv. *trifolii*. The mechanism generating these rearrangements is only poorly understood, although genetic instability appears to be an inherent property of these organisms.

From the initial investigations by Terzaghi (unpublished data), it appeared that Tn*5* may be responsible for triggering the plasmid rearrangement. Probing for Tn*5* revealed that it inserts preferentially into the plasmid doublet. Of the exconjugants screened, Tn*5* was never found in bands corresponding to the stable 600 kb and 370 kb plasmids.

These results strongly suggest that Tn*5* does not insert randomly into the genome of this organism. From Tn*5* mutagenic studies in other organisms random insertion of Tn*5* is expected. This means on a statistical probability basis, only 25% of transposition events in strain 2668 should involve the plasmids, and because the larger 600 kb and 370 kb plasmids present larger targets, these should be sites of

Tn*5* insertion more frequently than the two smaller plasmids. Thus, there is a serious discrepancy between the expected and the observed distribution of the Tn*5* insertion sites.

The aim of this study is to better characterise the observed Tn*5*-induced rearrangement as seen in strain *R. leguminosarum* 2668. But, the similar mobilities of the two plasmids making up the doublet made differentiation between them difficult, especially for interpretation of Southern blot analysis of Eckhardt gels. This problem is exacerbated by the lack of plasmid-specific probes to help differentiate between the plasmids. The need for an alternative bacterial model to study this apparent Tn*5*-induced rearrangement of plasmid profiles led to the selection of the partially characterised soil isolate OR168 (Jarvis *et al.*, 1989). OR168 was isolated from a white clover-ryegrass pasture and shows some chromosomal homology to *R. leguminosarum* 2668 (O'Hara, 1989). OR168 is able to accept the p*Sym* plasmid from *R. leguminosarum* 2668 via conjugative transfer. The resultant OR168::p*Sym* progeny show full phenotypic expression of genes encoding symbiotic characteristics. They are able to infect, nodulate and fix nitrogen in white clover (O'Hara, 1989). This suggests OR168 is either pSym-deficient *R. leguminosarum*, or some other closely related *Rhizobium* species that can accept and express the pSym plasmid.

Fortunately the OR168 isolate appears to be an ideal candidate to act as a model. It contains five stable episomal elements which are easily delineated using Eckhardt gel electrophoresis. Also, plasmid rearrangement with consequences similar to those observed with *R. leguminosarum* 2668 has been observed in OR168 when it was a recipient for conjugative plasmid transfer from a *R. leguminosarum* 2668::Tn*5* derivative (O'Hara,1989).

The principle aim of this study is to characterise more fully the consequences of the biparental conjugative transfer of the pSUP1011 vector containing Tn*5* from *E. coli* PN302 into OR168. This will provide a starting point for the systematic analysis of departures from expected results.

To determine the statistical significance of observed rearrangements upon the heterogeneric conjugative introduction of Tn*5* into the soil isolate OR168, two direct matings between OR168 and PN302 will be undertaken. A large number of exconjugants will be isolated from these crosses and the site(s) of Tn*5* insertion determined using Southern blotting analysis of Eckhardt gels. Hopefully this will shed light on the observed 'non-random' insertion of Tn*5* seen in 2668. This will determine the relative frequencies of Tn*5* insertion in each of the native replicons found in OR168. It may also determine whether it is due to the presence of

preferred insertion sites, or whether the observed results are due to a low sample number, coupled with the low frequency of transposition imposing a strong bias.

Total genomic digestion of DNA from selected exconjugants by selected restriction enzymes, followed by Southern blotting and probing of the electrophoretically separated fragments for various components of pSUP1011, will determine the total number of sites of insertion of pSUP1011 components present in the genome of screened exconjugants.

Further characterisation of the number and precise position of sites of insertion will be carried out using restriction enzyme analysis of selected exconjugants. The precise point of insertion of any pSUP1011 components present in the exconjugants of interest can be made using finer restriction mapping techniques. Sequence-specific DNA probes can be used to identify which of the components making up pSUP1011 are present in the exconjugant genome.

2. MATERIALS AND METHODS

2.1 Chemicals and Reagents

Agarose A6013 (Type I, low EEO), Chloramphenicol, CTAB (hexadecyltrimethyl ammonium bromide), Ethidium bromide, Lysozyme, Neomycin, Spectinomycin, N-Lauroyl sarcosine, Sodium dodecyl sulphate were obtained from Sigma Chemical Company.

Nylon membranes (Hybond-N, 0.45 μm) and RNase A powder (from bovine pancreas) were obtained from Amersham.

Proteinase K was obtained from BDH Ltd.

Cesium chloride was obtained from Varlacoid Chemical Company.

Sea plaque (low gelling temperature agarose) was obtained from FMC Bioproducts.

DNA labelling and detection kit - nonradioactive: Cat. No. 1093 657 was obtained from Boehringer Mannheim. This kit was used for random primed DNA labelling of probe DNA with digoxigenin-11-dUTP and for the subsequent colorimetric detection of DNA hybrids by enzyme immunoassay.

DIG Luminescent Detection Kit: Cat. No. 1363 514 was obtained from Boehringer Mannheim. This nonradioactive chemiluminescent system was used for the detection of digoxigenin-labelled probe DNA. Unlike the chromogenic (colorimetric) detection system, membranes can be easily reprobed when using this detection system.

DNA molecular weight marker II, digoxigenin-labelled Cat. No. 1218 590; Restriction endonucleases (*Bam*HI, *Eco*R1, *Hin*dIII); and positively charged nylon membranes were also obtained from Boehringer Mannheim.

All chemicals for buffer preparation and reagents were of analytical grade. All heat stable buffer solutions were autoclaved at 121°C for 15 minutes to sterilise before use.

Antibiotic stock solutions for both neomycin and spectinomycin were prepared by dissolving 1.0 g of the antibiotic powder in 200 ml of Milli-Q water, giving a final concentration of 5 mg/ml. The solutions were filter sterilised by passing through a Swinnex 25 mm disc filter holder containing a 0.22 μm pore size membrane filter (Millipore, type HA - autoclaved *in situ*).

Antibiotic stock solution for Chloramphenicol was prepared by dissolving 100 mg of the antibiotic in 20 ml of absolute ethanol, giving a final concentration of 5 mg/ml.

Stock solutions of all antibiotics were aliquoted and stored at -20°C until required and added aseptically to the media to selectively screen for organisms carrying the antibiotic resistance markers of interest. The final concentrations used are as listed in the methods. Typically, if the final concentration of the antibiotic was 50 μg/ml it meant diluting the stock solution 1/100 directly into the media.

2.2 Bacterial Strains and Plasmid Vectors

2.2.1 Characterisation of Bacterial Strains Used

1. *Escherichia coli* PN302:
This is a broad host range mobilising donor strain derived from *E. coli* C600 and used for the delivery of the transposon Tn*5*. It contains the *trans*-acting transfer genes from plasmid RP4 integrated into its chromosome. The Inc Pα plasmid RP4 has a very broad host range and is capable of transfer between, and stable maintenance in, almost all Gram-negative bacteria. RP4 also contains genes conferring resistance for ampicillin, kanamycin, and tetracyclin. The tetracycline resistance gene on the integrated RP4 replicon has been inactivated by insertion of the bacteriophage Mu. The ampicillin resistance gene has also been inactivated by a small deletion within Tn1.

PN302 also contains the mobilisable pSUP1011 plasmid vector (Simon *et al*, 1983a). This 12.6-kb plasmid consists of the 4.2-kb pACYC184 vector with a 2.6-kb *Sau*3A restriction fragment from RP4 containing the *oriT* site cloned into the unique *Bam*HI site within the tetracycline resistance gene. This has resulted in the insertional inactivation of the Tc^R gene. The 5.8-kb transposon Tn5 is contained within the still functional RP4 *oriT*-containing fragment.

pSUP1011 carries antibiotic resistance genes to chloramphenicol (100 μg/ml) from Tn9 (on pACYC184) and kanamycin (50 μg/ml) from Tn5. There is also a functional kanamycin resistance gene within the integrated RP4 DNA.

For purposes of clarity, throughout this thesis the term *oriT* (origin of transfer) will be used in reference to the specific *cis*-active DNA site essential for the conjugative transfer of plasmid DNA. It is at this site that the transfer process is initiated. For RP4, the minimal fragment mediating full *oriT* activity is approximately 350 bp in length and contains the *nic* site. The term RP4-*mob* will be used as originally defined by Simon *et al.*, (1983) in reference to the 2.6 kb *Sau*3A *oriT*-containing fragment of RP4 that was cloned into pACYC184 to create pSUP101. This RP4-*mob* fragment is thought to encode for a number of transfer-essential genes, as well as containing the *oriT* site.

Fine genetic mapping of the Tra1 region of RP4 (in which the *oriT* site is located) has revealed four gene products that are transcribed from two different operons. The divergent promoters of these two operons are located within the *oriT* site. Their gene products include the 11 kDa TraJ protein, the 15 kDa TraK protein, the 26 kDa TraL protein, and the 12 kDa TraM protein (Guiney & Lanka, 1989; Ziegelin *et al.*, 1992). Although the exact location of the 2.6 kb *Sau*3A *oriT*-containing fragment of RP4 has not been determined, it is sufficiently large to include at least some of the genes forming the two operons bounding the *oriT* site.

2. OR168:

This is a semi-characterised soil isolate from Jarvis *et al* (1989). It is a Gram-negative motile rod, from which a spectinomycin-resistant derivative has been isolated. It is this derivative of OR168 which was used in this study as a recipient for the transposon Tn5. The organism grows well on TY medium at 28-30°C with broth culture saturation (A_{600} >0.8) within 48-72 hours. Antibiotic selection at a final concentration of 50 μg/ml of spectinomycin was used to maintain the culture.

Comparison of the cell wall lipid components with other eubacteria, groups OR168 closely with *Rhizobia* species and in particular *Rhizobium meliloti*. This tentative phylogenetic classification has been supported by comparing the highly conserved 16S rRNA sequence of OR168 with those of other Gram-negative soil bacteria. The 16S rRNA sequence data of OR168 shows that it is closely related to, but distinct from, *Rhizobium meliloti* (Sivikumaran, PhD thesis in preparation).

3. DH101 - 127 and DH201 - 226:
These are the series of stable exconjugants isolated from two separate heterogeneric bacterial conjugations between OR168 and PN302. These isolates, like their wild-type parental strain (OR168), grow well on TY medium at 28-30°C and develop the same colony morphology. Antibiotic selection at a final concentration of 50 μg/ml spectinomycin was used to keep the cultures pure. To positively select for maintenance of the transposon Tn5, 50 μg/ml neomycin was used in the medium when growing these exconjugants.

4. *E. coli* PN334:
This strain contains the high copy number plasmid pKan2. This is pBR322 containing a 3.42 kb internal *Hin*dIII fragment of Tn5, cloned into the unique *Hin*dIII site. The *Hin*dIII fragment was used as a source of probe DNA for Tn*5* in Eckhardt gel Southerns, total genomic Southerns and slot-blots (Scott *et al.*, 1982). The plasmid was maintained by growing PN334 on LB medium containing 35 μg/ml chloramphenicol and 50 μg/ml kanamycin.

5. *E. coli* PN338:
This strain contains the high copy-number plasmid pSUP202. This 7.9 kb plasmid consists of the 6.0 kb pBR325 with a 1.9 kb IncPα-specific (RP4) *oriT*-containing *Sau*3A fragment cloned into a Sau3A site between the genes encoding resistance to chloramphenicol and tetracycline (Simon *et al*, 1983b). Maintenance of the plasmid was achieved by growing PN338 cells on LB medium containing 100 μg/ml ampicillin, 35 μg/ml chloramphenicol and 25 μg/ml tetracycline.

6. *E. coli* 402A:
This strain contains the 4.2 kb medium copy-number plasmid pACYC184. This plasmid carries the chloramphenicol-resistance gene from Tn9 and the tetracycline-resistance gene from pSC101. The plasmid was maintained by growing cells on LB media containing 35 μg/ml chloramphenicol and 25 μg/ml tetracycline.

2.2.2 Growth and Maintenance of Bacterial Cultures

All *E. coli* strains were grown in Luria-Bertani (LB) broth (Miller, 1972): (g/l) Tryptone, 10.0; Yeast Extract, 5.0; NaCl, 10.0; pH adjusted to 7.4 with 1 M NaOH. Any liquid media were solidified by the addition of 12 g/l Bacto-agar before sterilising. Media was sterilised by autoclaving at 121°C for 15 minutes. Saturated broth cultures (A_{600} >1.0) of *E. coli* grow in 24 hours when incubated at 37°C with shaking. Colonies are visible on solid medium after overnight incubation at 37°C.

The soil isolate OR168, all OR168 derivatives as well as other soil bacterial strains used were grown in Tryptone Yeast extract (TY) broth (Beringer, 1974): (g/l) Tryptone, 5.0; Yeast extract, 3.0. Both solid and liquid media were prepared and sterilised as above and allowed to cool below 55°C before adding 6 ml/l sterile 1 M $CaCl_2$. Cooling prevented calcium precipitates forming. The soil bacterial strains used are relatively fast growing, although appreciably slower than the *E. coli* strains used. Cultures of the soil bacterial strains grow to saturation (A_{600} >1.0) in 48 to 72 hours when incubated at 28°C with shaking. Colonies are visible on solid medium after incubation at 30°C for 3 days.

For the short term storage of all stock bacterial strains used, a single colony was inoculated into 5 ml of broth and grown to saturation with shaking. Single colonies were isolated by streak plating onto solid medium. Selection with the appropriate antibiotics was used for maintenance of pure cultures. Plates were sealed with parafilm and stored inverted at 4°C after colonies had grown up. Cultures were sub-cultured (on media with the appropriate antibiotics) every month to maintain their viability.

For stable long-term storage of all bacterial strains, saturated cultures were prepared as above. One volume (330 µl) of sterile Tris-buffered glycerol solution (65% (w/v) glycerol; 100 mM $MgSO_4$; 25 mM Tris, pH 8.0) was placed in sterile cryotubes (Nunc). Two volumes (660 µl) of completely resuspended, saturated culture was added to the glycerol solution. The tube contents were mixed by inversion and then snap-frozen in liquid nitrogen. The cultures can be stored indefinitely at -70°C.

Table 2.1: List of bacterial strains and plasmids used

Strain	Antibiotic Markers	Relevant comments source or ref
***E. coli* PN302**	$Cm^RSm^RKm^RNm^R$	contains the Tn5 donating suicide vector, pSUP1011 (pAYC184 + RP4 *oriT*::Tn*5*) Simon *et al.*, 1983a
***E. coli* PN338**	$Ap^RCm^RTc^R$	contains pSUP202 (pBR325 + RP4 *oriT*) Simon *et al.*, 1983b
***E. coli* 408A**	Cm^RTc^R	contains pACYC184 Chang & Cohen, 1978
***E. coli* PN334**	Ap^RTc^S	contains pKan2 (pBR322 with 3.4-kb inner *Hin*dIII fragment of Tn*5*) Scott *et al.*, 1982
***E. coli* V517 (PN1111)**	Km^R	contains 7 cryptic plasmids with size range of 1.9 kb to 50 kb, for plasmid size analysis Macrina *et al.*, 1978
OR168	Sp^R	partially characterised soil isolate Jarvis *et al.*,1989
DH101 - 127	$Sp^RKm^RNm^R$(Tn*5*)	OR168 x PN302 1st independent cross this study
DH201 - 226	$Sp^RKm^RNm^R$(Tn*5*)	OR168 x PN302 2nd independent cross this study
***Rhizobium meliloti* U45**		source of CCC plasmids for size analysis
***Agrobacterium tumefaciens* C58**		source of CCC plasmids for size analysis

2.3 Conjugation of Bacteria: OR168 x PN302 Crosses

Two separate heterogeneric conjugative transfers were carried out between *E. coli* PN302 donors and OR168 recipients using the procedures outlined below. These were carried out to introduce Tn*5* into OR168.

2.3.1 Preparation of Parental Strains

A single colony of OR168 was inoculated into 5 ml of TY broth containing 50 μg/ml spectinomycin and grown with shaking at 28°C for 48 hours. A 1/50 dilution from the resultant saturated culture was made and grown overnight in TY broth (no antibiotic) with shaking at 28°C. This culture served as the source of the recipient parental strain for the conjugation.

A single colony of PN302 was inoculated into 5 ml of LB broth containing 50 μg/ml kanamycin and grown overnight with shaking at 37°C. A 1/50 dilution from the resultant saturated culture was made and grown for three hours in LB broth (no antibiotic) with shaking at 37°C. This culture served as the source of the donor parental strain for the conjugation.

2.3.2 Conjugation Procedure

Three sterile filter discs (Millipore type HA, 25 mm diameter, 0.45 μm pore size) were placed on the surface of a warm fresh air-dried TY agar plate. 100 μl of mid-log growth phase PN302 was then pipetted onto one of the filter discs. After allowing time for the liquid to diffuse away, 100 μl of OR168 was pipetted onto the same filter disc. Each parental strain was also spotted separately onto one of the other filters as a check that both parental cultures were viable and as a source of cells for later control platings. The plate was left for 30 minutes in a laminar flow cabinet with the lid left ajar to allow the liquid to diffuse away before moving it. The plate was then inverted and incubated overnight at 30°C.

2.3.3 Recovery of Cross Progeny

Quantitative recovery of all viable cells involved in the mating (parental strains and progeny) was achieved by removing the filter and resuspending the cells growing on it by vortexing in 1.0 ml of sterile standard sodium citrate solution (1 x SSC: 150 mM NaCl; 15 mM sodium citrate, pH7.0).

The resuspended cells were diluted to 10^0, 10^{-2}, 10^{-5} and 10^{-6} in sterile SSC. 100 µl of each dilution was spread-plated onto selective media for enumeration of parental strains and cross progeny. The plates were incubated for 3 days at 30°C and the colonies counted.

2.3.4 Estimation of Frequency of Transposition Event

To enumerate putative exconjugants from each cross, TY Sp(50 µg/ml) Nm(50 µg/ml) agar was used as a selective medium. To enumerate the donors from each cross TY Nm(50 µg/ml) agar was used as a selective medium. Recipients were enumerated using TY Sp(50 µg/ml) as a selective media. Frequency of transfer of the antibiotic marker, neomycin resistance from PN302 to OR168 was calculated using the following relationship:

Frequency of transfer = number of exconjugants expressing both antibiotic markers per ml/number of recipients per ml.

2.3.5 Estimation of Frequency of Spontaneous Mutation

The frequency of spontaneous mutations in OR168 giving rise to neomycin-resistant progeny and in PN302 giving rise to spectinomycin-resistant progeny was also measured. The rate of these spontaneous events was calculated using the following relationship:

Frequency of spontaneous mutation = number of cells expressing resistance to both antibiotic markers per ml/number of cells expressing the respective parental antibiotic resistant phenotype per ml.

This was done by spread-plating 100 µl of each parental culture diluted to 10^0, 10^{-2}, 10^{-5} and 10^{-6} onto TY agar and TY Sp (50 µg/ml) Nm (50 µg/ml) agar, incubating for 3 days at 30°C then counting colonies.

2.3.6 Isolation of Exconjugants

After colonies had grown up (3 days at 30°C) on TY Sp (50 µg/ml) Nm (50 µg/ml) agar from the two matings, putative exconjugants were randomly selected from colonies showing similar morphology to OR168. Each selected isolate was individually picked off, vortexed briefly in 0.5 ml 1x SSC containing 0.1% (w/v) N-lauroylsarcosine to break up clumped cells. A loopful of the cell suspension was

streaked for single colonies on antibiotic-free TY agar and incubated at 30°C for 3 days. A single colony from each streak-plate was then streaked again onto antibiotic-free TY agar and incubated for 3 days at 30°C. A randomly chosen single colony was then spotted onto TY Sp (50 μg/ml) Nm (50 μg/ml) agar and incubated as before to check antibiotic resistance. This long process was undertaken for each isolate to ensure its purity.

Isolates showing the same colony morphology and growth characteristics as the parental donor, OR168, and expressing resistance to both antibiotic markers spectinomycin and neomycin, were regarded as genuine recombinant exconjugants.

2.4 Analytical Minigel Electrophoresis

This horizontal electrophoresis method was used for the quick analysis of DNA samples prior to further treatment. It was generally used to visually assay cleavage products after restriction endonuclease digestion of both plasmid and genomic DNA. This was to check digestion had gone to completion. The technique was also used to determine purity and yields of plasmid and total genomic DNA preparations. By running in parallel the appropriate size and/or concentration standards, the technique was also used to estimate the size and/or concentration of DNA preparations.

0.7% (w/v) agarose gel (in TBE buffer: Boric acid, 89 mM; EDTA, 2 mM; Tris, 89 mM; pH 8.0) was melted and cooled to 50°C before being poured into a minigel box apparatus with a well-forming comb. After setting, the gel was submerged in electrophoresis (TBE) buffer and the well-forming comb removed.

1 μl of DNA sample was added to 5 μl gel loading buffer (in TBE buffer: Bromophenol blue, 0.1%; Ficoll type 400, 15%; SDS, 0.25%; EDTA, 10 mM; pH 8), mixed well and transferred to a well in the gel. The high density of the loading buffer allows for easy loading of DNA samples into the submerged wells of the gel. The presence of tracking dye allows progress of the electrophoresis to be monitored. The SDS inhibits enzyme activity.

Electrophoresis was carried out at room temperature at 100 V until the bromophenol blue dye front reached within 2 to 3 cm of the end of the gel (approximately 90 minutes). The dye front co-migrates with linear DNA fragments of about 200 bp. The gel was removed from the gel box and stained and photographed (as in Section 2.6).

2.5 Horizontal Eckhardt Gel Electrophoresis

Eckhardt gel electrophoresis is a technique developed by Eckhardt (1978) for the *in-situ* examination of large extrachromosomal elements existing freely within prokaryotic cells. The major advantage of this technique over other methods used for plasmid preparation (such as the 'rapid boil' technique) is that because the plasmids are not exposed to excessive hydrodynamic shear, they are extracted from the cells in their native form. The *in situ* lysis of the intact cell minimises handling of the DNA, reducing the chance of breakage.

2.5.1 Reagents and Solutions

Tris-Borate-EDTA buffer (TBE): 89 mM Tris; 89 mM Boric acid; 2.5 mM EDTA; pH 8.2

Tris-EDTA buffer (10/1 TE buffer): 10 mM Tris; 1 mM EDTA; pH 8.0.

RNase A solution (DNase activity free): 20 mg of RNase A powder was dissolved in 2.0 ml of 10/1 TE buffer, then boiled for 2 minutes to remove DNase activity. The solution was stored at -20°C.

Solution #1 (in TBE buffer): 10% (w/v) Ficoll type 400; 0.05% (w/v) Bromophenol blue; 0.3 U/ml RNase A (DNase activity free); lysozyme (freshly added) 1/10 volume of 2 mg/ml solution in TBE. The solution was stored at +4°C and warmed to room temperature prior to use.

Solution #2 (in TBE buffer): 10% (w/v) Ficoll type 400; 0.2% (w/v) SDS; 4.0 mg/ml Proteinase K. The solution was stored at -20°C and warmed to room temperature prior to use.

Solution #3 (in TBE buffer): 5% (w/v) Ficoll type 400; 0.2% (w/v) SDS. The solution was stored at +4°C and warmed to room temperature prior to use.

2.5.2 Agarose Gel Preparation

An agarose gel was prepared at least four hours prior to use. This is thought to allow for extensive cross-linking to occur as the gel hardened. This procedure reduced band distortions during electrophoresis. A 0.7% (w/v) agarose gel was prepared using agarose A6013 (Type 1, low EEO, Sigma) in TBE buffer. The suspension was boiled until the solution clarified, then cooled to 55°C before casting in a horizontal gel apparatus.

2.5.3 Sample Preparation and Electrophoresis

A single colony was inoculated into 5.0 ml of TY broth and incubated with shaking at 28°C for 48 hours. A 5.0 ml subculture of this saturated culture was prepared (by diluting 1/100 in fresh TY) and incubated with shaking at 28°C for 18 hours. This ensured a source of log-phase cells. The appropriate antibiotics were added to the media to maintain selection for the strain. 1.0 ml of the 18 hour culture was pipetted into a 1.5 ml Eppendorf tube and centrifuged in a microfuge at 12000 rpm for 3 minutes. The supernatant was carefully pipetted off and the cell pellet resuspended completely in 1.0 ml of 10/1 TE buffer. The cells were centrifuged again for 3 minutes and the supernatant removed as before.

The cell pellet was resuspended in 20 µl of Solution #1 by drawing up the contents of the tube and expelling gently 2-3 times using a micropipette. The cell suspension was then immediately transferred to a blot-dried well in the gel. The transferred cells were incubated at room temperature for 15 minutes to allow the lysozyme to degrade the cell wall. 20µl of Solution #2 was added to the well. Incubation at room temperature was continued for 15 minutes to allow the SDS to disrupt the cell membrane and the Proteinase K to digest proteinaceous material before overlaying with solution #3.

The gel was submerged by filling the electrophoresis tank with TBE buffer. Electrophoresis was carried out at 20 volts for one hour (0.6 V/cm) and then at 90 volts for 15 hours (3 V/cm) at 4°C. Progress and direction of DNA migration can be monitored by following the bromophenol blue dye front which migrates in the electric field. At pH 8.0 DNA migrates towards the cathode (positive pole).

2.6 Gel Staining and Photography

125 mg/ml Ethidium bromide stock solution was diluted to 25 µg/ml to visualise DNA on electrophoretic gels. The ethidium bromide intercalates with the DNA helix and DNA-ethidium bromide complexes will fluoresce when exposed to UV light. Gels were removed from the electrophoresis tank, immersed in a solution of ethidium bromide for 15 minutes, rinsed in tap water for 30 minutes and then placed on a short-wave UV transilluminator and photographed at f8 for a 2 second exposure using Polaroid 667 instant film through a Wrattan 23A red filter.

Photographic negatives were made by exposing Kodak Tmax 400 film for 40 seconds at f22. The film was developed by complete immersion in D19 developing solution with gentle mixing for 5 minutes, followed immediately by a 30 second rinse in distilled water, then complete immersion in fixative with gentle mixing for a further 5 minutes. The film was then rinsed under running tap water for a further 5 minutes before air drying.

The luminescence emitted by the decomposition of AMP~D, resulting from the alkaline phosphatase-catalysed dephosphorylation of AMPPD, was documented by exposing X-ray film (Fuji RX film) for 20 minutes at room temperature to the treated membranes (See Section 2.12.3.2 for details regarding membrane treatment). The exposed X-ray film was developed and fixed as for photographic negatives above. The developed X-ray film documenting Southern hybridisation results detected with the DIG Luminescent detection system is known as a lumigraph.

2.7 DNA Isolation and Purification Methods

The following section outlines the common protocols for DNA isolation and purification used in the study.

2.7.1 Rapid Isolation of Plasmid DNA

The rapid boiling method used for recovering plasmids from bacteria is based on that of Holmes and Quigley (1981). It is suitable for plasmids up to 35 kb, but efficiency of recovery declines with increase in size.

2.7.1.1 Reagents for rapid isolation of plasmid DNA.

STET Buffer: 8% (w/v) sucrose; 5% (w/v) Triton-X100; 50 mM EDTA; 50 mM Tris, pH 8.0.

Lysozyme Solution: 10 mg/ml lysozyme in 10 mM Tris pH 8.0 (25 µl aliquots stored at -20°C).

Cold (-20°C) isopropanol.

70% ethanol at room temperature.

2.7.1.2 Procedure

A colony of cells containing the plasmid of interest was inoculated into LB broth and grown overnight (stationary phase) with shaking at 37°C. 1.0 ml of this culture was pelleted using a microfuge. The supernatant was removed and the cell pellet resuspended completely in 350 µl of STET buffer. 25 µl of lysozyme solution was added to the cell suspension and mixed thoroughly. The tube was allowed to sit for 5 minutes before being placed in a boiling water bath for 40 seconds. The tube was immediately spun for 10 minutes in a microfuge at 12000 rpm. The gelatinous plug of chromosomal and cellular debris was removed with a toothpick. The plasmid DNA in the remaining supernatant was then precipitated with an equal volume of cold isopropanol, left at -70°C for 30 minutes, then centrifuged for 30 minutes at +4°C. The supernatant was decanted and discarded. The DNA pellet was washed once with 1.0 ml of 70% ethanol at room temperature, drained well and dried under vacuum using a Speedvac apparatus. The DNA was resuspended in 50 µl 10/1 TE buffer and stored frozen (-20°C) until required.

2.7.2. Phenol-Chloroform Extraction of DNA

The DNA in DNA-protein mixes can be further purified by treatment with phenol. Contaminating protein will preferentially associate with the organic phenol phase, or at the interface, while DNA will preferentially associate with the aqueous phase. Phenol is equilibrated with 10 mM Tris, pH 8.0 before use. 2-Hydroxy-quinoline at 0.1% (w/v) is used as an antioxidant.

An equal volume of Tris-equilibrated phenol was added to the DNA preparation; vortexed, to ensure phases remain mixed for 5 minutes; then centrifuged at 12000 rpm for 5 minutes at room temperature in a microfuge to separate phases. The top aqueous phase was recovered, avoiding the white interface, and transferred to a fresh tube. The aqueous supernatant is then extracted with an equal volume of phenol-chloroform (1:1 v/v) as before. This step was repeated if no clear phase separation was seen due to a high concentration of protein at the interface. A final extraction in an equal volume of chloroform was made. The aqueous phase was collected as

above. This process produced DNA ready for ethanol precipitation and washing (see Section 2.7.3).

2.7.3 Ethanol Precipitation and Washing of DNA

To precipitate DNA, 0.1 volumes of 3 M sodium acetate, pH 5.2 was added, mixed well, then 3 volumes of cold (-20°C) 95% ethanol was added and mixed well (alternatively 0.6 volumes of cold isopropanol can be used instead of the ethanol). The solution was left to stand overnight at -20°C to precipitate (alternatively left at -70°C for a minimum of 30 minutes). The DNA solution can be left indefinitely at this stage of purification. After allowing the DNA to precipitate, it was pelleted by centrifuging for 30 minutes at 12000 rpm and +4°C. The supernatant was then removed and discarded. The pellet was washed to remove residual salt and phenol by carefully overlaying with 500 µl 70% ethanol at room temperature and centrifuged for 15 minutes. The supernatant was removed and discarded. Tubes were then placed in a Speedvac for 5 minutes to remove residual ethanol. The DNA was resuspended in an appropriate solvent to the required concentration.

2.7.4 Measurement of DNA Concentration and Purity

The concentration of DNA solutions was calculated by measuring their absorbency value in a UV spectrophotometer (Shimadzu UV-160A) at wavelength 260 nm against a solvent blank. Generally the solvent was 10/1 TE buffer (10 mM Tris; 1 mM EDTA; pH 8.0). The DNA sample was usually diluted 1/100 in the solvent. The following equation was used to calculate DNA concentration:

$$[\text{DNA}] = A_{260} \cdot 50 \cdot \text{dilution factor} \quad \mu g/ml$$

where 50 is the absorptivity constant of dsDNA at 260 nm,
and A_{260} is the absorbance at 260 nm in a 1 cm cuvette.

The purity of the DNA solutions was estimated by comparing their absorption ratios at 260 nm and 280 nm (A_{260}/A_{280}). Pure solutions of DNA have an A_{260}/A_{280} ratio of 1.8. Anything significantly less than this figure indicates protein or phenol contamination, while anything significantly greater than this figure indicates RNA contamination. Accurate measurement of DNA concentration is severely compromised if the A_{260}/A_{280} ratio differs significantly from 1.8.

2.8 Southern Blotting Analysis

The technique of Southern blotting (Southern, 1975) allows for the detection of specific DNA sequences in a complex mixture of DNA molecules. For this purpose the DNA molecules were separated according to size by gel electrophoresis, denatured by alkali treatment, transferred to a solid membrane support and immobilised by heating or UV cross-linking.

A VacuGene XL blotting unit (Pharmacia-LKB) was used for the transfer of DNA to either nylon (Hybond N, Amersham) or positively charged nylon (Boehringer Mannheim) membranes. Using the vacuum blotting apparatus, the pretreatment and transfer of DNA on gels was completed within 3 hours.

If Southern blots were to be reprobed, charged nylon membranes were used for the transfer and the DNA fixed by UV cross-linking. Also, the membranes were kept wet at all stages from prehybridising to stripping to prevent problems with high background staining or lowered sensitivity.

2.8.1 Solutions for Vacuum Blotting

Depurination solution: 0.25 M HCl
Denaturation solution: 1.5 M NaCl, 0.5 M NaOH
Neutralising solution: 1.0 M Tris, 2.0 M NaCl, pH 5.0
Transfer solution: (20 x SSC) 3 M NaCl, 0.3 M sodium citrate, pH 7.0

2.8.2 Preparation of the Blotting System

A mask was prepared by cutting a 'window' slightly smaller than the gel in plastic sheeting. This allowed an overlap of 5 mm between the gel and the mask and ensured adequate sealing during blotting.

A membrane, cut to the same size as the gel, was pre-wetted in Milli-Q water and then immersed in transfer solution for 15 minutes. The blotting apparatus was then assembled with membrane, mask and gel. A constant vacuum pressure of 55 kPa is used throughout.

2.8.3 Blotting Procedure

2.8.3.1 Depurination

The gel was carefully flooded with the depurination solution which was drawn through the gel under vacuum until the bromophenol blue indicator dye in the gel turned yellow (usually 20 minutes). This treatment results in the random hydrolysis of a few purine bases at their deoxyribose moiety.

Plasmid DNA preparations separated by Eckhardt gel electrophoresis were treated for 40 minutes with the depurination solution. This was found to be necessary to generate DNA fragments small enough to allow quantitative transfer of the large plasmid molecules during the succeeding transfer procedure.

2.8.3.2 Denaturation

The gel was denatured by alkali treatment for the same length of time as the depurination step. This treatment following low pH depurination causes DNA cleavage at apurinic sites and separation of the DNA duplex strands. Only ssDNA will bind to the membrane support and is able to anneal to specific probe sequences when probed later.

2.8.3.3 Neutralisation

The gel was then neutralised with high salt Tris buffer for 30 minutes. The high salt buffer aids DNA binding to the membrane.

2.8.3.4 Transfer

Transfer of the DNA on the gel was achieved using 20 x SSC as solvent and a slight vacuum as a driving force. The transfer of the DNA to the filter retains the pattern of DNA bands originally seen in the gel. The rate of transfer is dependent on the buffer salt concentration, gel thickness and fragment size. One hour was sufficient to achieve quantitative transfer of DNA from both Eckhardt and normal gels. Gels were restained with ethidium bromide to check that this had happened. The membrane was immersed in 2 x SSC for 2 minutes with gentle shaking before being blot-dried and fixed. The DNA was irreversibly fixed to the nylon membrane surface by baking under vacuum (80°C, -80 kPa) for 2 hours. DNA was fixed to positively charged nylon membranes by baking for 15 minutes at 120°C or, alternatively, fixed by UV cross-linking at 254 nm for 3 minutes after the membrane had been completely air-dried. The fixing process also allows for the convenient storage of membranes until required for DNA hybridisation with labelled probes.

2.9 Nucleic Acid Slot-Blots

The slot-blot technique provides a rapid qualitative screening method for the presence of specific DNA sequences in total genomic DNA preparations. It is less time consuming than Southern blot analysis with direct transfer of the DNA. A multi-well filtration manifold (Schleicher and Schüll) was used to obtain even application of the DNA samples to the nylon membrane support without the problem of their lateral spreading.

Total genomic DNA extracts were prepared as outlined in section 2.10.1. After determining DNA concentration of the extracts (see section 2.7.4), 1.0 µg aliquots were resuspended in a total volume of 50 µl of TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). Then 0.1 volume (5 µl) of 3 M NaOH was added and the tubes incubated in a water bath at 65°C for 1 hour to denature the DNA. The tubes were then cooled to room temperature and 1 volume (55 µl) 2 M NH_4OAc, pH 7 added.

The slot-blot manifold was assembled according to the instructions supplied by the manufacturer with both the 2 sheets of 3MM filter paper (Whatman) and the nylon membrane, being cut to size and prewetted in 1 M NH_4OAc, pH 7 before being placed on the filtration manifold. A low vacuum was applied and the wells of the manifold washed with 500 µl of 1 M NH_4OAc, pH 7 before quantitatively transferring the denatured DNA samples to individual wells. Each well was rinsed with a further 500 µl of 1 M NH_4OAc, pH 7 before dismantling the apparatus and recovering the membrane. The membrane was fixed using methods detailed in Section 2.8.3, then stored dry until required for DNA hybridisation with labelled probes.

Because of difficulties incurred by stripping blots after probing with colorimetric detection systems, when repeat probing of the same samples was required, replica blots were prepared. This simply meant multiplying amounts and volumes by the number of blots being prepared. After completing a blot preparation, the manifold was washed carefully, reassembled and a further aliquot of the stock samples was used to prepare replica blots.

2.10 Extraction, Digestion and Electrophoresis of Total Genomic DNA

The protocol detailed below is a hybrid of two different methods for obtaining total genomic DNA from *Rhizobium* species. It is a quick method for the isolation of relatively pure, digestible genomic DNA, without the need of CsCl gradients. The genomic DNA preparations were free of inhibiting contaminants which could interfere with restriction endonuclease activity. Typical yields were 2.5 μg to 7.0 μg DNA per 1.0 ml starting culture. The protocol is also suitable for isolating genomic DNA from *E. coli* strains where yields in excess of 50 μg DNA per 1.0 ml were achieved.

Using the method of Fisher & Lerman (1979) to obtain total DNA from strains OR168 and the OR168::Tn5 exconjugants yielded DNA that could not be cut cleanly by restriction endonucleases. The inhibition was thought to be due to the presence of contaminating exopolysaccharides which, even with prewashing of the cultures with 0.1% N-lauroylsarcosine, were not effectively removed.

The miniprep method of Wilson (1990) results in co-precipitation of RNA with the genomic DNA. The presence of RNA causes an over-estimation of the DNA concentration and must consequently be removed.

2.10.1 Isolation of Total Genomic DNA

A single colony was picked from the plate and inoculated into 10 ml of TY broth. The culture was grown to saturation (72 hours at 28°C for OR168 and OR168::Tn5 strains; 24 hours at 37°C for *E. coli* strains) with shaking and with antibiotic selection. The culture was transferred to a 10 ml centrifuge tube and cells were pelleted at 3000g (4200 rpm) for 15 minutes at room temperature using a Haereus Megafuge centrifuge. The supernatant was discarded and the cell pellet was resuspended in 1.0 ml 50/20 TE (10 mM Tris, 1 mM EDTA, pH 8.0) buffer containing 0.1% N-lauroylsarcosine. The cell suspension was transferred to a 1.5 ml Eppendorf tube and centrifuged for 3 minutes at 12000 rpm in a microfuge.

The supernatant was again discarded and the cell pellet was resuspended in 1.0 ml of 50/20 TE buffer to wash cells, centrifuged as before and the cells finally resuspended in 545 μl of 50/20 TE buffer. 10 μl of lysozyme (10 mg/ml in 50/20 TE buffer) and 5 μl of RNase A (10 mg/ml in 50/20 TE buffer; DNase activity free) were added, mixed well and the cell suspension incubated at 37°C for 60 minutes to break down

any RNA present. Then 10 µl of Proteinase K (20 mg/ml in 50/20 TE buffer) and 30 µl of 10% SDS were added and mixed well. Cellular digestion was continued at 50°C overnight (for 12 to 18 hours). The resulting cell lysate is very viscous and clear.

100 µl of prewarmed (65°C) 5 M NaCl was added to the lysate and mixed well by vortexing. This step is critical to prevent precipitation of CTAB-DNA complexes. The lysate was preheated to 65°C in a water bath before 80 µl of CTAB/NaCl solution [10% (w/v) CTAB in 0.7 M NaCl, prewarmed to 65°C] was added and mixed thoroughly. The tubes were then incubated at 65°C for a further 10 minutes. Then an approximately equal volume (0.7 to 0.8 ml) of chloroform was added and the tube contents mixed thoroughly by vortexing. The tubes were then centrifuged at 12000 rpm for 10 minutes to separate phases. The CTAB-protein/polysaccharide complexes are seen as a dense white precipitate at the interface. The viscous aqueous supernatant was then transferred to a fresh tube. Phenol-chloroform extraction of the DNA was then carried out (see Section 2.7.2) followed by ethanol precipitation (see Section 2.7.3).

2.10.2 RE Digestion and Electrophoretic Fragment Separation

After vacuum drying, the DNA was dissolved in 50 µl of sterile 10/1 TE buffer and stored frozen (-20°C) until required. Under these storage conditions, the DNA preparations were stable. The concentration of the purified genomic DNA preparations were estimated by spectrophotometric determination (see Section 2.7.4) and 2 µg aliquots placed in 1.5-ml Eppendorf tubes. Digestion of genomic DNA was carried out overnight (15 hours) at 37°C using 5.0 units of restriction endonuclease/µg of DNA and the appropriate restriction buffer conditions in a total reaction volume of 50 µl.

The following restriction endonucleases were used to cleave the genomic DNA samples - both individually and in double RE digests: *Bam*HI, *Eco*RI and *Hin*dIII. The optimum buffer conditions for both *Bam*HI and *Hin*dIII are the same (10 mM Tris, 5 mM $MgCl_2$, 100 mM NaCl, 1 mM β-Mercaptoethanol, pH 8.0, 37°C). The same buffer conditions are suitable for both *Bam*HI/*Eco*RI and *Eco*RI/*Hin*dIII double RE digests, although the optimum buffer composition for *Eco*RI cleavage is 50 mM Tris, 10 mM $MgCl_2$, 100 mM NaCl, pH 7.5, carried out at 37°C.

Minigel electrophoresis was used to analyse the yield of DNA (see Section 2.4) and to check that digestion had gone to completion. If digestion was complete the reaction was stopped by adding 5 µl of 10 x SDS dye mix (0.25% bromophenol blue,

15% Ficoll type 400, 2% SDS, 100 mM EDTA, pH 8) followed by heating the mixture for 5 minutes at 65°C. For both single and double enzyme digests carried out on the genomic DNA, it was essential that digestion went to completion to avoid errors when later counting and sizing bands showing up on probed Southern blots.

The cleavage products of RE-digested genomic DNA were separated using agarose gel electrophoresis. Typically 1.0 μg (25 μl) of digested product was loaded per well of a 0.7% agarose gel (Type I, low EEO in TBE buffer). Before loading samples, the gel was submerged to a depth of 1 to 2 mm with electrophoresis buffer (TBE buffer: Boric acid, 89 mM; Tris, 89 mM; EDTA, 2 mM, pH 8.0). Electrophoresis was carried out overnight (18 hours) at 40 volts (1.5 V/cm) at room temperature in a BioRad DNA Sub Gel tank. The gel was stained and photographed (see Section 2.6) before Southern blotting (see Section 2.8).

2.11 Preparation of Probe DNA

Four different plasmid-derived DNA sequences were used as probes to detect homologous sequences on Southern and dot blots.

The DNA used to probe for transposon Tn5 was the internal 3.5-kb *Hin*dIII fragment of Tn5 which had been cloned into the unique *Hin*dIII site on pBR322. This plasmid construct is referred to as pKan2 (Scott *et al.*, 1982).

The DNA used to probe for the IncPα-specific replicative origin of DNA transfer (RP4-specific *oriT*) was the 3.1 kb *Bam*HI/*Eco*RI fragment excised from the pSUP202 vector construct (Simon *et al.*, 1983a, 1983b).

Purified plasmids pACYC184 (Chang & Cohen, 1978) and pSUP1011 (Simon *et al.*, 1983a) were also labelled and used as probe DNA.

The DNA used as probe had to be purified and linearised prior to carrying out the random-primed labelling reaction (see Section 2.12.1)

2.11.1 Large-Scale Preparation of Plasmid DNA

The following protocol describes the methods used to obtain large amounts of purified DNA from existing vector sequences for use as probe DNA. The first step involves alkaline lysis to obtain a crude plasmid DNA preparation followed by isopycnic CsCl/EtBr gradient centrifugation to separate the plasmid DNA from chromosomal DNA and RNA. This second step is based on the differential ability of these nucleic acid molecules to bind EtBr. Binding EtBr increases the buoyant density of the DNA. Supercoiled (covalently closed circular) plasmid DNA does not have the capacity to bind as much EtBr/unit length as linear chromosomal fragments which results in the plasmid DNA having a lower buoyant density than linear DNA. Plasmid banding occurs lower in the tube than chromosomal banding. RNA appears as a diffuse band at the bottom of the tube.

2.11.1.1 Preparation of Crude Lysates

The *E. coli* strain containing the required vector sequence was grown up overnight in 5 ml LB broth with shaking at 37°C. Antibiotic selection was used to exert selective pressure for plasmid maintenance. This culture was inoculated into 500 ml of LB broth and grown to saturation (approximately 24 hours) with vigorous shaking at 37°C. The cells were harvested by centrifuging at 8000 rpm, 4°C using a Sorvall GSA rotor. The cells were washed by resuspending in 100 ml of Tris-EDTA buffer: 10 mM Tris; 1 mM EDTA; pH 8.0 (10/1 TE buffer), followed by recentrifuging at 8000 rpm.

The cells were resuspended in 8 ml glucose-Tris-EDTA solution (50 mM glucose, 25 mM Tris, 10 mM EDTA, pH 8.0), transferred to SS-34 tubes before adding 1 ml lysozyme (50 mg/ml in glucose-Tris-EDTA solution), and left to stand for 10 minutes at room temperature. 10 ml freshly prepared NaOH/SDS solution (0.2 M NaOH, 1% SDS) was added and mixed gently by inversion, then placed on ice for 20 minutes. 8.9 ml potassium acetate solution (To 60 ml of 5 M potassium acetate add 11.5 ml glacial acetic acid and 28.5 ml H_2O. The final solution is 3 M with respect to potassium and 5 M with respect to acetate.) was added and mixed by inversion before standing on ice for a further 10 minutes. The resultant precipitate was pelleted by centrifuging for 10 minutes at 13000 rpm, 4°C in a Sorvall SS-34 rotor.

Most of the chromosomal DNA along with SDS-protein complexes and other cellular debris is contained in the pellet. The supercoiled plasmid DNA remains in the supernatant. The supernatant was transferred to a clean tube and the DNA precipitated by adding 0.6 volumes cold (-20°C) isopropanol, mixing by inversion, then leaving at room temperature for 20 minutes. The DNA was recovered by

centrifuging for 10 minutes at 12000 rpm, room temperature using a Sorvall SS-34 rotor. The pellet was washed with 20 ml 70% ethanol at room temperature, then recentrifuged and the supernatant discarded. The residual ethanol was removed under vacuum and the dried DNA pellet resuspended in 4.5 ml 10/1 TE buffer. The DNA can be stored frozen (-20°C) indefinitely or further purified using CsCl/EtBr equilibrium centrifugation (Section 2.11.1.2).

2.11.1.2 Isopycnic Cesium Chloride/Ethidium Bromide Centrifugation

The DNA pellet from the final step of the crude lysate preparation (Section 2.11.1.1) was completely resuspended in 4.5 ml 10/1 TE buffer. To this 4.77 g CsCl (1.06 g/ml of DNA solution) was added and dissolved. Then 337.5 µl of 10 mg/ml ethidium bromide stock was added and the solution mixed well. It was then left to stand overnight at 4°C and EtBr-protein complexes and other precipitates removed by centrifuging for 10 minutes at 12000 rpm, 4°C using a Sorvall SS-34 rotor. The solution was then transferred to a 5-ml ultracentrifuge tube and centrifuged for 5 hours at 55000 rpm, 17°C using a Sorvall combi TV865 rotor.

The banded plasmid DNA was usually visible in the gradient in normal daylight, but occasionally side illumination with low intensity longwave UV light was needed. Plasmid DNA was recovered from the tube by inserting a 20-G needle into its top (to provide an air inlet) and inserting a second 20-G needle with a 2-ml syringe attached just below the plasmid band and drawing it off. The EtBr was removed from the recovered plasmid DNA by extracting 3 to 4 times with equal volumes of 10/1 TE saturated butanol. The CsCl was removed by dialysing against 500 to 1000 vol 10/1 TE buffer at 4°C with 4 changes of buffer every 6 to 12 hours.

The purity and yield of plasmid DNA was checked using minigel electrophoresis (see Section 2.4) and measuring the A_{260}/A_{280} ratio spectrophotometrically (see Section 2.7.4).

2.11.2 Small-Scale Preparation of Plasmid DNA

This alternative protocol describes the methods used to obtain small amounts of purified DNA from existing vector sequences for use as probe DNA without the need to use CsCl/EtBr gradient steps.

2.11.2.1 Isolation of DNA

15 ml of overnight cultures of the *E. coli* strains carrying the required plasmid were grown up with shaking at 37°C on LB broth. Antibiotic selection was used to ensure plasmid maintenance. Plasmid DNA was recovered using the rapid boil technique

(10 1-ml aliquots were processed; see Section 2.7.1). The DNA pellet in each tube was resuspended in 50 µl 10/1 TE buffer and allowed to dissolve overnight at 4°C. The resuspended DNA was pooled into 2 1.5-ml Eppendorf tubes (250 µl/tube) then treated with 5 µl RNase A (10 mg/ml; DNase activity free) for 30 minutes at 37°C prior to phenol-chloroform extraction (see Section 2.7.2) and ethanol precipitation (see Section 2.6.3).

The purified DNA pellet was resuspended in 50 µl 10/1 TE buffer. The purity and yield of plasmid DNA was checked using minigel electrophoresis (see section 2.4) and measuring the A_{260}/A_{280} ratio spectrophotometrically (see Section 2.7.4).

2.11.2.2 Excision and Recovery of Probe DNA Sequence

If the entire plasmid vector was to be used as probe DNA (as in the case of pSUP1011 and pACYC184), 50 µg of purified plasmid DNA (obtained either as per Sections 2.11.1.2 or 2.11.2.1) was linearised using *Eco*RI at 5 units/µg of DNA in a total reaction volume of 500 µl. This RE cleaves both plasmids at a unique site. Digestion was carried out for 2 hours at 37°C. A minigel (see section 2.4) was run to check for complete digestion of the DNA alongside uncut DNA samples and DNA size ladders. When digestion was complete the reaction was stopped by adding 1/10 vol 0.2 M EDTA. The linearised DNA was recovered by phenol-chloroform extraction (Section 2.7.2) followed by ethanol precipitation (Section 2.7.3). The DNA pellet was dried under vacuum before resuspending in 50 µl 10/1 TE buffer.

If the required probe DNA was only a fragment of the plasmid vector (as in the case of Tn*5* probe from pKan2 [a 3.4 kb *Hin*dIII fragment] and RP4-specific *oriT* probe from pSUP202 [a 3.1 kb *Eco*RI/*Hin*dIII fragment]) RE digests were carried out as below to obtain the fragment of interest.

50 µg of purified plasmid DNA (obtained either as per Sections 2.11.1.2 or 2.11.2.1) was cleaved using each restriction endonuclease at 5 units/µg of DNA in a total reaction volume of 500 µl. Digestion was carried out for 2 hours at 37°C. A minigel (see section 2.4) was run to check for complete digestion of the DNA alongside uncut DNA samples and DNA size ladders. When digestion was complete the reaction was stopped by adding 50 µl 10x SDS loading dye.

A 7 mm thick 0.7% seaplaque agarose gel in TAE buffer (Tris, 40 mM; Na acetate, 20 mM; EDTA, 1 mM; pH 7.2 with acetic acid) was made using an Horizon 11.14 gel box (BRL) and well-forming comb to produce an 11 cm by 14 cm gel with a central well of dimensions 1 mm wide, 6 mm deep and 92 mm long which could accommodate 550 µl of sample. The restriction products were separated by gel

electrophoresis at 25 V, 4°C (18 hours) in TAE buffer alongside *Hin*dIII λ digest and BRL 1-kb size markers.

The gel was then stained in ethidium bromide and viewed under longwave UV. The band corresponding in size to the required product was excised from the gel using a scalpel blade. The DNA was recovered from the agarose (Koenen, 1989) by loading the excised gel plug into sterile 0.5-ml Eppendorf tubes with the lid removed and with a hole made in the bottom with a 21-G needle. To prevent the agarose being forced through the hole during centrifugation, a wad of siliconised glass wool was hard-packed into the bottom of the tubes before loading with the agarose plug. The tubes were then set in 1.5-ml Eppendorf tubes and spun for 5 minutes at room temperature, 6500 rpm in a microfuge. This results in compression of the agarose and the buffer-containing-DNA is forced through the hole in the bottom of the Eppendorf tube. The DNA-containing supernatant that collected in the 1.5-ml Eppendorf tube was pooled and the DNA recovered using high salt-isopropanol precipitation (see Section 2.7.3). After drying under vacuum the purified DNA pellet was resuspended in a total volume of 50 µl 10/1 TE buffer.

The purity and concentration of purified probe DNA was checked using minigel electrophoresis (see Section 2.4) and measuring the A_{260}/A_{280} ratio spectrophotometrically (see Section 2.7.4).

2.12 Probe DNA Labelling, Hybridisation and Detection

Nonradioactive labelling and detection systems were used to probe Southern and slot blots prepared from DNA samples transferred to nylon membrane supports (see Sections 2.8 and 2.9). The systems used throughout this study were developed by Boehringer Mannheim (Cat. Nos. 1093 657 and 1363 514). Preliminary trial runs of the hybridisation and detection steps were used to optimise conditions for both nonradioactive labelling and detection systems. Using the protocols in this section, the labelling and detection systems gave good specificity of hybridisation and sensitivity of detection. The principles and methods for each are detailed below.

2.12.1 Labelling Probe DNA

The linearised purified probe DNA was labelled with the nucleotide analog, digoxigenin-11-dUTP (dig-11-dUTP) using the random primed labelling technique of Feinberg and Vogelstein (1983). This initially involved denaturing 150 ng of the prepared probe DNA (Section 2.11) by boiling for 10 minutes. It was then plunged into ice cold water to prevent reannealing of the DNA. Complete and efficient denaturation is essential for effective labelling. Complementary strand synthesis was initiated with incorporation of dig-11-dUTP occurring every 20 - 25 nucleotides, using 2 µl (1 µM) random hexanucleotide primer and 1 µl (2 units) Klenow enzyme, with the addition of 2 µl dNTP labelling mixture (dATP, 1 mM; dGTP, 1 mM; dCTP, 1 mM; dTTP, 0.65 mM; dig-11-dUTP, 0.35 mM). Complementary strand synthesis was carried out at 37°C for 20 hours.

The reaction was stopped by the addition of 2 µl 0.2 M EDTA, pH 8.0; then the DNA was precipitated using high salt-ethanol precipitation (see Section 2.7.3) to remove non-incorporated dNTPs and dig-11-dUTP. After drying under vacuum, the digoxigenin-labelled probe DNA was resuspended in 50 µl H_2O.

The rate of *de novo* synthesis of labelled DNA decreases with increasing dig-11-dUTP:dTTP ratio. The rate of synthesis obtained using dig-11-dUTP alone without dTTP is 1/3 less than the rate obtained using unlabelled dTTP. The best sensitivity is obtained using a dig-11-dUTP:dTTP ratio of 35%. Higher ratios also result in steric hindrance between the digoxigenin label and the antibody conjugate used to detect the label.

The conditions used for labelling were those recommended by Boehringer Mannheim, the manufacturer of the kit.

2.12.2 Hybridisation of Labelled DNA to Target DNA

Before hybridising the labelled probe to the immobilised target DNA, the membranes were prehybridised. This pretreatment prevents non-specific binding of probe DNA to the membrane by blocking such sites with hybridisation buffer solution. This buffer contains N-lauroylsarcosine, 0.1% (w/v); SDS, 0.02% (w/v); plus the blocking reagent (vial 11 from kit) 1% (w/v), in 5x SSC solution.

Because the blocking reagent contains fractionated milk proteins, it dissolves slowly. Hybridisation buffer was prepared fresh 1 hour in advance by dissolving at 68°C in a water bath. The membrane prepared by Southern transfer (see Section 2.8 for

procedure) was sealed flat in a plastic bag with all air expelled, and with at least 20 ml of hybridisation solution per 100 cm^2 of membrane. Prehybridisation was carried out for 2 hour at 68°C in a shaking water bath.

The prehybridisation solution was then discarded and replaced with fresh hybridisation solution (about 2.5 ml/100 cm^2 of membrane) containing 150 ng of freshly denatured (boiled for 10 minutes then plunged into ice cold bath to prevent reannealing) labelled probe DNA. The bag containing the membrane was resealed, excluding all air bubbles and submerged flat in a shaking water bath at 68°C overnight (18 hours).

The membranes were then washed 2x 5 minutes at room temperature with at least 50 ml of wash solution 1 (2x SSC; SDS, 0.1% w/v) per 100 cm^2 membrane and 2x 5 minutes at 68°C with wash solution 2 (0.1 x SSC; SDS, 0.1%, w/v). The membranes can now be used directly for detection of hybridised DNA or stored air-dried.

Hybridisation was carried out overnight using low stringency conditions to obtain sequence-specific binding between the labelled probe DNA and the target DNA. The post-hybridisation washes were carried out under more stringent conditions and this step was used to control the amount of non-identical sequence hybridisation.

Stringency is dependent on a number of hybridisation parameters, such as pH, salt concentration, temperature and the use of organic solvents. Stringency can be easily modified by altering these parameters. It is expressed in terms of °C below the calculated melting temperature (T_m) of the probe-target DNA hybrid. T_m can be calculated using the following equation, and is used as an indication of duplex stability (Meinkoth and Wahl, 1984):

$$T_m = 81.5 + 16.6(\log[Na^+] + 0.41[\%\ (G+C)] - 500/n$$

where $[Na^+]$ is the concentration of monovalent ions (generally sodium ions),
%(G+C) is the proportion of guanine and cytosine residues in the probe DNA,
and n is the length of hybrid molecule (in bp).

For the hybridisation conditions used, stringency was T_m - 28°C. This compares to a stringency of T_m - 16°C for the wash steps.

Stringency can be easily increased by decreasing salt concentration. This was done at the washing steps to eliminate non-homologous sequences and sequences with only partial homology forming hybrids. If left unchecked, non-identical sequence hybridisation can make interpretation of results difficult.

2.12.3 Detection of Probe-Target DNA Hybrids

Two different systems were used to detect probe-target DNA hybrids. With both systems, detection involved the binding of a polyclonal alkaline-phosphatase conjugated antibody to digoxigenin moieties present on the digoxigenin-labelled hybrid DNA.

Alkaline phosphatase enzyme at pH 9.5 converts the soluble colourless substrates BCIP [5-**B**romo-4-**c**hloro-2-**i**ndolyl-**p**hosphate] and NBT [4-**N**itro **b**lue **t**etrazolium chloride] into insoluble chromogenic products which are visualised as blue/brown precipitates on the membrane. At pH 9.5 the enzyme also dephosphorylates the chemiluminescent substrate AMPPD [3-(2′-Spiro**a**damantane)-4-**m**ethoxy-4-(3″-**p**hosphoryloxy)-**p**henyl-1,2-**d**ioxetane] to the chemically unstable intermediate, AMP~D. The intermediate slowly decomposes via dioxethane fragmentation giving off light that can be detected using X-ray film (analogous to autoradiograms). Using positively charged nylon membranes, blots can be easily stripped of probe and rehybridised.

2.12.3.1 Chromogenic Detection System

The membrane was rinsed briefly (1 min) in 200 ml/100 cm^2 of Buffer 1 (Tris-HCl, 100 mM; NaCl, 150 mM; pH 7.5) at room temperature with shaking, then washed for 30 minutes with shaking in 200 ml/100 cm^2 of Buffer 2 (Buffer 1 with 0.5% (w/v) blocking reagent, prepared 1 hour in advance by dissolving at 50-70°C). The membrane was rinsed again briefly in Buffer 1 before sealing in a plastic bag. 20 ml/100 cm^2 of dilute anti-digoxigenin AP-conjugate [diluted to 150 mU/ml (1/5000) in Buffer 1] was added.

It was then incubated at room temperature with gentle shaking for 30 minutes. Unbound antibody-conjugate was removed by washing 2x 15 minutes with 200 ml/100 cm^2 Buffer 1 followed by equilibration in 20 ml/100 cm^2 of Buffer 3 (Tris, 100 mM; NaCl, 100 mM; $MgCl_2$, 50 mM; pH 9.5) for 5 minutes at room temperature.

The membrane was then resealed in a bag with 10 ml/100 cm^2 of freshly prepared colour solution (45 µl NBT solution (vial 4); 35 µl BCIP solution (vial 5) in 10 ml Buffer 3), and it was left undisturbed in the dark to allow for colour development (colour precipitate).The reaction was stopped by washing the membrane for 5 minutes with 10/1 TE buffer. Results were documented by photocopying the wet filter.

2.12.3.2 Chemiluminescent Detection System

The membrane was rinsed briefly (1 min) in 200 ml/100 cm^2 of Buffer 1 (Tris-HCl, 100 mM; NaCl, 150 mM; pH 7.5) at room temperature with shaking, then washed for 30 minutes with shaking in 200 ml/100 cm^2 of Buffer 2 (Buffer 1 with 0.5% (w/v) blocking reagent, prepared 1 hour in advance by dissolving at 50-70°C). The membrane was rinsed again briefly in Buffer 1 before sealing in a plastic bag. 20 ml/100 cm^2 of dilute anti-digoxigenin AP-conjugate [diluted to 75 mU/ml (1/10000) in Buffer 1] was added.

It was then incubated at room temperature with gentle shaking for 30 minutes. Unbound antibody-conjugate was removed by washing 2x 15 minutes with 200 ml/100 cm^2 Buffer 1 followed by equilibration in 20 ml/100 cm^2 of Buffer 3 (Tris-HCl, 100 mM; NaCl, 100 mM; $MgCl_2$, 50 mM; pH 9.5) for 5 minutes at room temperature.

The membrane was then resealed in a bag with 10 ml/100 cm^2 of dilute AMPPD solution (10 mg/ml stock diluted 1/100 in Buffer 3) and shaken gently for 5 minutes at room temperature. The substrate solution was poured off the membrane, then the excess removed by placing it in a plastic bag on a flat surface and rolling with a 10-ml glass pipette. The damp membrane was sealed in the bag, then incubated at 37°C for 15 minutes followed by a 30 minute incubation at room temperature to allow a build-up of AMP~D before exposing to X-ray film for 20 minutes. The film was then developed as outlined in section 2.6. The diluted AMPPD substrate solution was stored in the dark at 4°C and reused up to 5 times.

2.12.3.3 Stripping and Reprobing Southern Blots

The chemiluminescent detection with the DIG system was adopted because of the simple steps required for preparing Southern membranes for reprobing. The stripping of probe DNA was essential to remove all digoxigenin present. This was done by initially rinsing the membrane in sterile Milli-Q water. At no time was the membrane allowed to dry out. The membrane was then placed in a large dish and 1 litre of boiling 0.1% SDS solution poured on to it. The solution was allowed to cool to room temperature with gentle mixing before discarding and repeating the wash. The membrane was then rinsed in 2x SSC before repeating the detection step to ensure removal of all probe. With no evidence of residual digoxigenin, the membrane was rinsed in 2x SSC before repeating the prehybridisation step as a prerequisite for the next probe hybridisation.

2.13 Screening of Exconjugants for Antibiotic Resistance Markers

Phenotypic expression of a plasmid-borne antibiotic resistance marker is indicative of a plasmid bearing that gene being present in that exconjugant isolate. The 'suicide' plasmid pSUP1011 used for the delivery of Tn*5* into OR168 contains the antibiotic resistance marker to chloramphenicol. The OR168 recipient is sensitive to chloramphenicol. The expected loss of pSUP1011 from the exconjugants was shown using the following antibiotic resistance testing protocol. The presence of pSUP1011 would make the exconjugants Cm^R.

2.13.1 Determination of the Bactericidal Concentration of Chloramphenicol

A culture of OR168 was grown to saturation in TY Sp(50 μg/ml) medium by incubating at 30°C for 48 hours with shaking. Then 1/100 subcultures were prepared in fresh TY Sp(50 μg/ml) media containing the antibiotic chloramphenicol over a range of concentrations from 0 to 50 μg/ml. These subcultures were incubated at 30°C for 72 hours with shaking. Increasing the incubation time beyond 72 hours in the presence of the antibiotic only increased the risk of selecting spontaneous Cm^R mutants. After 72 hours a 100 μl aliquot of each subculture was spread-plated onto a fresh TY Sp(50 μg/ml) agar plate. They were incubated up to 5 days at 30°C to verify the presence of viable cells. The extra-long incubation was to allow for resuscitation of any cells simply inhibited by the antibiotic. It was thus determined that concentrations of chloramphenicol below 20 μg/ml were only bacteriostatic for OR168. Above 20 μg/ml chloramphenicol was bactericidal. A concentration of 30 μg/ml was used to ensure 100% bactericidal action of the antibiotic against OR168 and any non-resistant exconjugant strains.

2.13.2 Screening of Exconjugants for Antibiotic Resistance Markers

Cultures of each exconjugant and the OR168 recipient were grown to saturation in TY Sp(50 μg/ml) medium by incubating at 30°C for 48 hours with shaking. Then using patch plating, a loopful of each culture was spotted onto fresh TY Sp(50 μg/ml) agar plates containing either chloramphenicol at 30 μg/ml or Neomycin at 50 μg/ml or no other antibiotic. The plates were incubated at 30°C for 5 days to verify the presence of viable cells. Resistance to the antibiotics resulted in visible growth within 2 days.

3. RESULTS

3.1 Creation and Isolation of OR168::Tn*5* Derivatives

The aim of the first set of experiments was to randomly tag the plasmids native to OR168 with the transposable element Tn*5*. The initial purpose of this experiment was that once tagged with the transposable element any subsequent movement of Tn*5*-containing plasmids between bacteria could be monitored by following the transfer of kanamycin resistance, which is associated with Tn*5*.

The transposon Tn*5* was successfully introduced directly into the genome of the soil isolate OR168 using the suicide plasmid technique of Simon *et al.* (1983a). The mobilising *E. coli* donor strain PN302 was used for the conjugative transfer of Tn*5* (carried on the pSUP1011 plasmid vector) into OR168. PN302 contains the *trans*-acting transfer function genes of the broad host-range plasmid RP4 integrated into its chromosome. This allows for the mobilisation of pSUP1011 (which is a pACYC184 derivative containing the cloned *mob* site from RP4) into the recipient cell OR168.

It is assumed the pSUP1011 vector is not stably maintained in the recipient because its origin of replication is not recognised by the new host. The failure of pSUP1011 to replicate means that cross progeny showing neomycin resistance (expressing aminoglycoside 3′-phosphotransferase) are assumed to have undergone a transposition event. The expected result is the random insertion of Tn*5* into a site within the OR168 genome. Because the initial matings and subsequent isolation procedures were carried out on complex growth media (TY agar) even insertions resulting in the creation of auxotrophic mutations should be isolated. Only lethal mutations cannot be recovered.

Quantitative recovery of all viable cells involved in the mating (parental strains and progeny) was achieved by resuspending cells present on the filter in SSC solution and plating out 100µl aliquots at different concentrations onto appropriate media. The results of the antibiotic selection screening of the cross progeny are shown in Table 3.1.1. The figures for each cross represent the average colony count for two separate platings on the selection media indicated.

The wild-type recipient OR168 strain is sensitive to low concentrations of the aminoglycosides kanamycin and neomycin. But OR168 is resistant to relatively high concentrations (50μg/ml) of the aminocyclitol antibiotic spectinomycin. Because of this it can be selectively grown in media containing this antibiotic.

The donor strain, *E. coli* PN302 is sensitive to low concentrations of spectinomycin, but shows high resistance to the aminoglycosides kanamycin and neomycin (50μl/ml) because of the expression of the *kan* gene within Tn*5*. It can be selectively grown in media containing either of these aminoglycosides.

The cross progeny that exhibit both spectinomycin and neomycin-resistance are assumed to be the soil bacterium, OR168, which has acquired a Tn*5* element from pSUP1011, following introduction of this plasmid from *E. coli* PN302. The frequency of this event was calculated to be approximately 5×10^{-4} per recipient (see Table 3.1.2).

The transfer frequency is 10-fold higher than that observed by Simon *et al.* (1983a) for similar matings between *E. coli* SM10 (that is *E. coli* PN302) and *R. meliloti*, and more than 10^5 fold higher than the frequency for spontaneous mutations giving rise to neomycin-resistant OR168 cells (see Table 3.1.3 for results). Based on these figures, the probability that exconjugant isolates are spontaneous parental mutants is remote (expect 1 per 5×10^4 isolates).

Two separate matings were carried out between OR168 and PN302 with OR168::Tn*5* progeny (exconjugants) selected on TY Sp(50) Nm(50) agar. After the colonies had grown up from the two matings putative exconjugants were randomly selected using the method detailed in Section 2.3.6. This selection method was used to ensure isolate purity and avoid contamination from overgrowth of naturally occurring mutants in the mixed background of cells present on the plates.

The isolation method also removed the risk of contamination from viable, but bacteriostatically suppressed parental cells. The removal of the antibiotic selection in subsequent culturing of exconjugants sets up the possibility they could be overgrown by suppressed cells and the exconjugants lost.

Cross progeny isolated using this method and showing the same colony morphology and growth characteristics as the parental recipient, OR168, as well as expressing high resistance to both antibiotic markers (spectinomycin and neomycin), were regarded as legitimate exconjugants. Each exconjugant isolated from both crosses was kept separate and formed the basis of the two series of isolates. These consisted

of the DH100 series (27 exconjugants from Cross 1) and the DH200 series (26 exconjugants from Cross 2).

The principle reason for identifying the cross origin of each exconjugant was to have some means of measuring the incidence and location of observed rearrangements within each cross. This also allows for comparisons of the frequency of observed rearrangements to be made between the two crosses, and hence some measure of the frequency of particular genetic events can be made.

Table 3.1.1: Isolation of progeny from OR168 x PN302 crosses

Cross Progeny	Selection Medium	Cell counts (cfu/ml)	
		Cross 1	Cross 2
Exconjugants	TY Sp(50) Nm(50)	3.9×10^3	4.2×10^3
Recipients	TY Sp(50)	6.0×10^6	1.0×10^7
Donors	TY Nm(50)	9.6×10^7	1.2×10^8
Total Count	TY	3.0×10^8	3.6×10^8

Table 3.1.2: Incidence of putative transposition events from crosses

Mating	Selection Media	Cell Count (cfu/ml)	Transposition Frequency
Cross 1	TY Sp(50) Nm(50)	3.9×10^3	6.5×10^{-4}
	TY Sp(50)	6.0×10^6	
Cross 2	TY Sp(50) Nm(50)	4.2×10^3	4.2×10^{-4}
	TY Sp(50)	1.0×10^7	

Table 3.1.3: Spontaneous marker mutations in parental strains

Organism	Selection Media	Cell Count (cfu/ml)	Frequency of Spontaneous Mutation
OR168	TY Sp(50)	1.4×10^9	$<7.1 \times 10^{-10}$
	TY Sp(50) Nm(50)	0	
PN302	TY Nm(50)	7.0×10^9	$<1.4 \times 10^{-10}$
	TY Nm(50) Sp(50)	0	

3.2 Determination of Plasmid Sizes for OR168 and Selected Exconjugants

Eckhardt gel electrophoresis shows the *Rhizobium*-like strain OR168 contains four cryptic plasmids (see lane # 5 of Eckhardt gel in Figure 3.2.1). These plasmids are of unknown size. Eckhardt gel electrophoresis also shows that exconjugants from the heterogeneric cross between OR168 and *E. coli* PN302 have altered plasmid profiles (see Section 3.3 and Figures 3.3.1a to 3.3.6a). An estimation of the plasmid sizes would be useful in determining the relative amounts of DNA involved in the plasmid rearrangements observed after the introduction of Tn*5*. It would also be useful to know the size of other plasmid species present in the exconjugant population. The aim of this exercise was to estimate plasmid size so that a measure of the magnitude of change in plasmid size as a result of the rearrangements could be made. Is the alteration in plasmid mobility simply due to the acquisition of the transposon Tn*5* by specific plasmids? Or, have more extensive rearrangements been incurred?

Plasmid size was determined by calibration against the relative mobilities of plasmids of known size. *Agrobacterium tumefaciens* C58 carries two plasmid species: pTi is 195 kb, pAt is 416 kb. *R. meliloti* U45 carries one plasmid: pRme is 153 kb (Casse *et al*, 1979). *E. coli* V517 carries eight plasmid species: pVA517a is 54.2 kb, pVA517b is 7.3 kb, pVA517c is 5.6 kb, pVA517d is 5.1 kb, pVA517e is 3.9 kb, pVA517f is 2.8 kb, pVA517g is 2.6 kb, and pVA517h is 2.0 kb (Macrina *et al*, 1978). Plasmids from these bacteria were used as size standards.

The size of plasmids for 4 exconjugants, along with the plasmids of the parental OR168 strain, were estimated using Eckhardt gel electrophoresis. Each bacterial strain used in the Eckhardt gel was prepared from cultures at mid-log phase growth using the method detailed in Section 2.5. The Eckhardt gel shown in Figure 3.2.1 displays the plasmids of the bacteria used as size standards along side the plasmids of the parental strain, OR168, and the exconjugants DH113, DH119, DH201, and DH216. The exconjugants DH113, DH201, and DH216 were also included in the plasmid size calibrations because accurate sizing of their 'd' plasmids (see below for explanation of 'd') was considered useful for the restriction mapping carried out in Section 3.8. DH119 was included purely as a check, since it appeared to have the same plasmid profile as DH113.

Cryptic plasmids have been named to easily identify their origin. The plasmid name is derived from the carrier strain and is prefixed with the letter 'p' to signify that it is 'plasmid'. For each strain the letter 'a' designates the least-mobile plasmid. The more

mobile plasmids of each strain are designated sequentially with the letters 'b', 'c', and 'd', in order of increasing mobility.

Figure 3.2.2 shows a plot of $\log_{10}$ plasmid size against relative mobility for the plasmids of known size (in kb). The electrophoretic mobility of pRme was set arbitrarily at 10 units and the mobility of the other plasmids was set relative to this. Fig 3.2.2 shows a consistent inverse linear relationship between $\log_{10}$ plasmid size and relative mobility: the larger the plasmid, the slower the rate of migration.

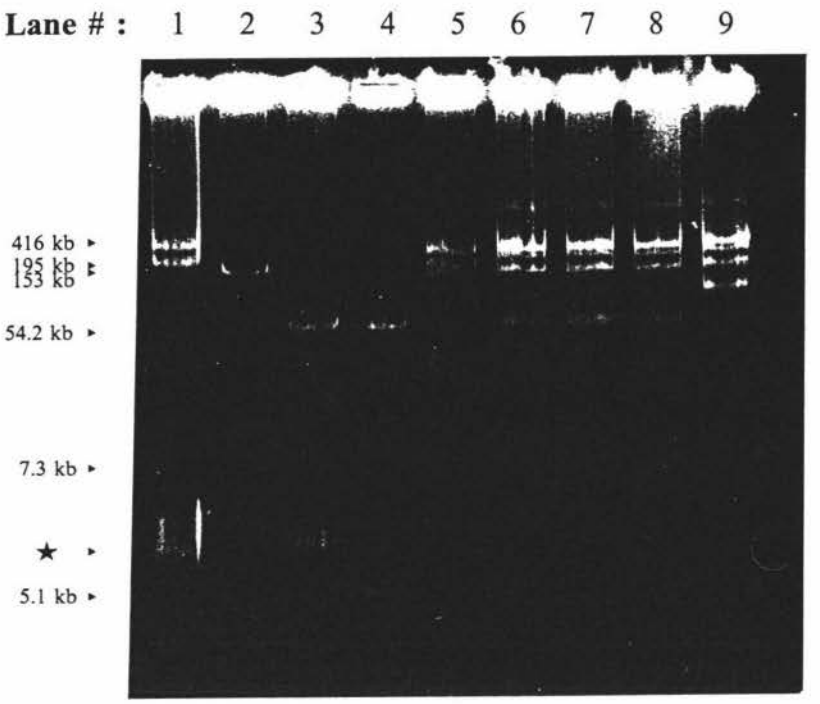
Extrapolations from Fig 3.2.2 give the estimated sizes of plasmids seen for OR168 and the exconjugants displayed in the Eckhardt gel of Figure 3.2.1. The resultant plasmid sizes are listed below in Table 3.2.1.

TABLE 3.2.1: Plasmid size determinations

STRAIN	PLASMID NAME	RELATIVE MOBILITY (RM)	PLASMID SIZE (kb)
A. tumefaciens C58	pAt	8.62	416
	pTi	9.47	195
R. meliloti U45	pRme	10.00	153
E. coli V517	pVA517a	12.77	54.2
	pVA517b	20.48	7.3
	pVA517c	26.60	5.6
	pVA517d	27.13	5.1
OR168	pOR168m	7.73	~1000
	pOR168a	8.40	570
	pOR168b	9.31	225
	pOR168c	9.84	163
	pOR168d	15.43	22
DH113	pDH113	7.73	~1000
	pDH113a	8.40	570
	pDH113b	8.94	295
	pDH113c	9.84	163
	pDH113d	13.03	40
DH119	pDH119m	7.73	~1000
	pDH119a	8.40	570
	pDH119b	8.94	295
	pDH119c	9.84	163
	pDH119d	13.03	40
DH216	pDH216m	7.73	~1000
	pDH216a	8.40	570
	pDH216b	8.94	295
	pDH216c	9.84	163
	pDH216d	11.06	65

NOTE: Table 3.2.1 lists the sizes of plasmid bands visible on the Eckhardt gel shown in Figure 3.2.1.

Figure 3.2.1: Eckhardt plasmid profiles of reference strains with known plasmid sizes compared with plasmid profiles of OR168 and 4 exconjugants with plasmids of unknown size.

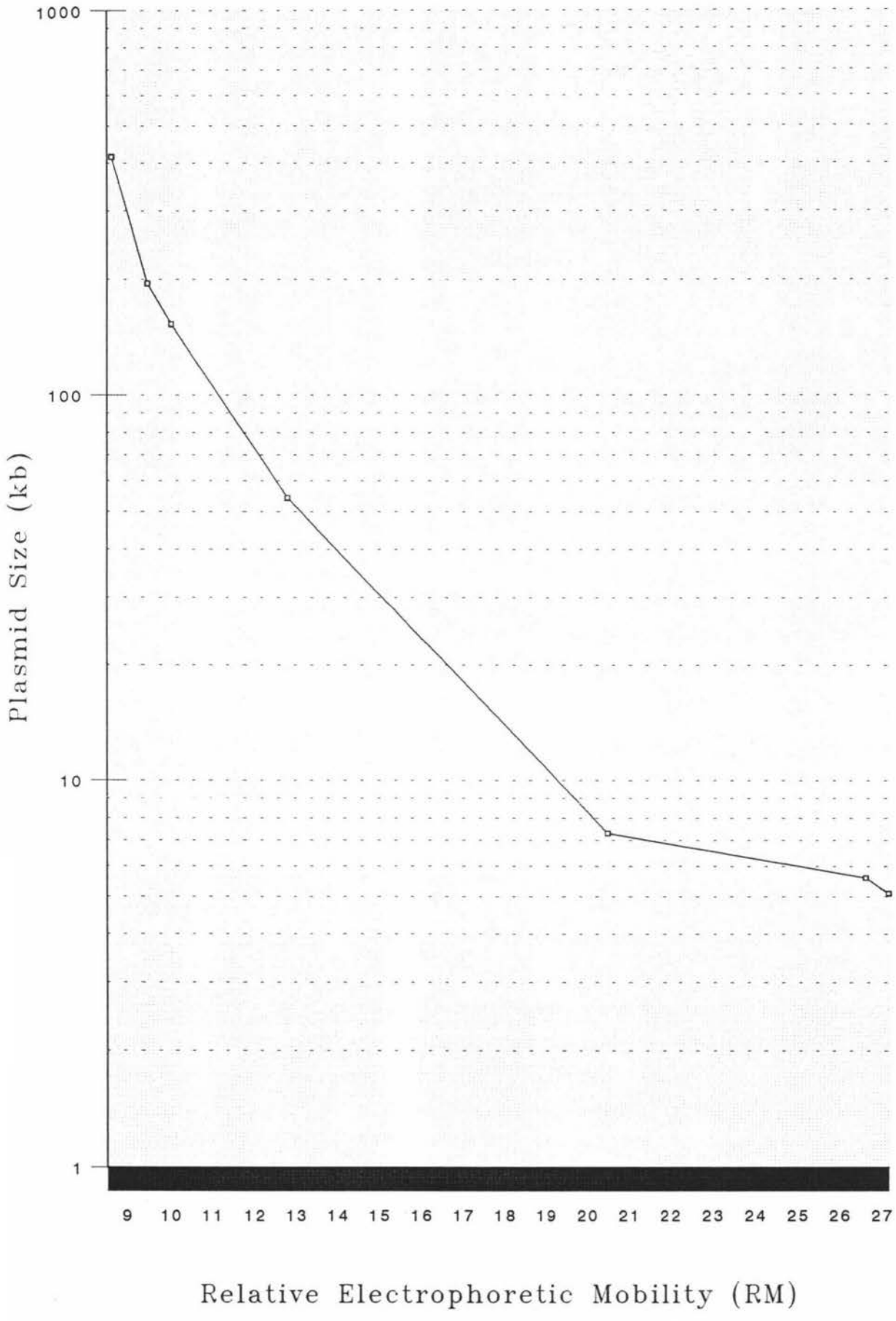


KEY:

Lane # :
1 *Agrobacterium tumefaciens* C58
2 *Rhizobium meliloti* U45
3 *Escherichia coli* V517
4 *Escherichia coli* V517
5 OR168
6 DH113
7 DH119
8 DH201
9 DH216

★ Chromosomal DNA 'artifact'

Figure 3.2.2: Semi-$\log_{10}$ plot of plasmid size against RM for determining plasmid sizes of exconjugants and OR168 using Eckhardt gel from Fig 3.2.1



Note: RM data obtained from gel shown in Figure 3.2.1

3.3 Comparison of Plasmid Profiles

3.3.1 Eckhardt Gel Electrophoresis Results

Horizontal Eckhardt gel electrophoresis was carried out on each of the 53 exconjugants isolated from two separate crosses. This was to facilitate the comparison of plasmid sizes and patterns for each exconjugant. The resulting plasmid profiles are shown in Figures 3.3.1a to 3.3.6a. The wild-type parental strain, OR168 was included on each gel as a reference.

After staining the gels in Figs 3.3.1a to 3.3.6a with ethidium bromide, DNA can be identified as intense fluorescent bands at the top of the gel corresponding with the wells, along with 3 to 4 distinct bright bands in the middle, and in some cases a faint uniformly wide band at the bottom. The intensity of the staining is directly proportional to the amount of DNA present. In a number of the lanes, a faint distinct band can be seen above the brightly staining bands in the middle of the gel.

These observations suggest the top band probably consists of intact chromosomal DNA and large open circular (OC) plasmid DNA which cannot migrate through the gel. Because of its much larger hydrodynamic radius, an OC plasmid migrates at a much slower rate than its CCC plasmid counterpart through the agarose gel. The brightly fluorescent bands in the middle of the gels are thought to be covalently closed, circular (CCC) plasmid bands. The faint bands which are occasionally visible above the least-mobile CCC plasmid band are probably megaplasmid molecules (see Section 3.3.3). Their apparent transient presence on the gels is probably an indication of their fragility in the lysis procedure used with Eckhardt gel electrophoresis to recover intact CCC plasmid molecules. The physical size of the megaplasmids results in them being more susceptible to any action which will cause them to fail to maintain perfectly their CCC conformation.

A comparison of the resulting plasmid profiles obtained for each exconjugant shows a large variation in band pattern relative to that seen for the wild-type OR168. This is evident as changes in plasmid mobility on the gel. A meaningful comparison of the plasmid bands can be made using the OR168 parent as an internal reference, even with differences in electrophoretic voltage and running time for each gel.

On all gels the wild-type OR168 shows five supercoiled plasmid bands producing a consistent profile pattern, and in most cases a megaplasmid is also present. The relative electrophoretic mobility (RM) of these plasmids has been calculated so that a

direct comparison of band mobilities between different gels can be made. Each plasmid band has been named for clarity in their identification.

From the results presented in Section 3.2, the sizes of the plasmids present in OR168 were estimated as being as follows:

pOR168m	~1000 kb
pOR168a	570 kb
pOR168b	225 kb
pOR168c	163 kb
pOR168d	22 kb

The OR168 plasmid complement now provides a set of known size standards for estimating the sizes of all plasmids visible on the gel photographs in Figs 3.3.1a to 3.3.6a. For this purpose the relative electrophoretic mobility (RM) of pOR168a was arbitrarily set at 10 units for each gel. Fortuitously, pOR168a appears to have very stable mobility, with a plasmid of the corresponding size being seen in each of the exconjugants. For determining plasmid sizes, the RM of all other plasmids on the gel was set relative to pOR168a. The RM values for each of the OR168 plasmids are consistent between the six gels, as is the RM value of the least mobile CCC plasmid for each exconjugant. For simplicity in this exercise, the megaplasmid has been ignored; primarily because it cannot be sized accurately, and secondly because it is not always clearly visible on the Eckhardt gels.

Tables 3.3.1 to 3.3.6 list figures for distance migrated (DM), relative mobility (RM) and estimated plasmid size (in kb) for all the supercoiled plasmid bands seen in Figures 3.3.1a to 3.3.6a inclusive. The semi-$\log_{10}$ plots of $\log_{10}$ plasmid size against RM for each gel are shown in Figure 3.3.11. The resultant straight lines display the consistent inverse semi-$\log_{10}$ relationship between plasmid size and mobility. Fundamentally, the smaller the plasmid molecule, the greater the distance it will migrate. For the purposes of this investigation, the explicit assumption has been made that all plasmids in the various derivatives have the same relative degree of supercoiling.

The first striking observation on comparing plasmid profiles is that of the 53 isolates screened none show the profile typical of the wild-type OR168 parental recipient. This implies a high incidence of plasmid rearrangement involving large segments of DNA, and in many cases involving more than one plasmid in the isolate.

A number of questions spring to mind on analysing these plasmid profiles. The foremost question is what role, if any, has Tn*5* played in these rearrangements? Prior to the introduction of Tn*5* into the OR168 genome its plasmid profile appeared

stable. Is the observed change in plasmid mobility solely due to the acquisition of a Tn*5* element? How many copies of Tn*5* are present in each of the exconjugants? If there is more than one copy of Tn*5* then how stable is the resultant plasmid complement? As the phenotypic expression of the *kan* gene has been used to select for exconjugants the presence of Tn*5* is assumed by conjecture. However its precise position and number within the altered genome of the exconjugants cannot be determined solely from the plasmid profiling exercise. It is hoped these questions will be answered fully during this study.

Because of the limited sensitivity of the Eckhardt gel system used, a change in electrophoretic mobility due to the insertion of the 5.8-kb Tn*5* element cannot be readily detected in the larger plasmids of OR168. Considering accurate measurement of all distances on the gels is ±0.5 mm at best, then differences only greater than 0.5 mm can be considered clear measurable changes in mobility. Any lesser distance difference could have resulted from variations in plasmid migration during the electrophoresis or even resulted from inaccuracies inherent in the limitations of the method used for measuring distances.

For instance, for the 570-kb plasmid, a difference in mobility of ±0.5 mm equates to a change in size of ±80 kb. Even for the 163-kb plasmid a change in mobility of ±0.5 mm equates to a change in size of ±12 kb. This means transposition events involving the larger plasmids of OR168 cannot be determined with any accuracy by simply comparing plasmid profiles.

In comparison, for the 22-kb plasmid a change in mobility of ±0.5 mm equates to a change in size of ±1 kb. This means a transposition event involving this plasmid as recipient can be detected by comparing plasmid mobilities. The addition of the 5.8-kb Tn*5* DNA to pOR168d is predicted to alter the RM value of the plasmid by more than 1 unit.

The measured alterations in plasmid mobility of the larger plasmids suggests the observed rearrangements involve DNA segments greatly in excess of 5.8 kb in many instances. This indicates events other than transposition have occurred. From the Eckhardt gel results alone, the only candidate where a possible incidence of Tn*5* transposition involving a plasmid target has occurred is that of DH120. Here it is assumed the 28-kb pDH120d has derived from the 22-kb pOR168d. The addition of 6 kb of DNA can be accounted for by the insertion of Tn*5* DNA.

Another explanation for altered plasmid mobility could be that a change in the degree of supercoiling of the native plasmids has taken place with the presence of Tn*5*. The alterations to plasmid profiles seen here typify those observed with the heterogeneric

conjugation between *R. leguminosarum* and *E. coli* PN302 (O'Hara, 1989). In both instances these rearrangements can be characterised grossly as the appearance or disappearance of particular bands, and the alteration in size of others. Variations in plasmid profile have also been observed with rhizobia populations isolated from the field.

Of the exconjugants screened, all but one clearly showed five supercoiled plasmid bands on the Eckhardt gels. The only exception, DH214, consistently shows four bands.

To ensure the observed plasmid profiles were reproducible, that is stable, the Eckhardt electrophoresis was repeated using the exconjugants of interest after many further generations of growth. The gel results for this are shown in Figures 3.3.7a to 3.3.9a and show that generally the plasmid profiles are unaltered after 12 months of culturing the exconjugants. The only noticeable exception involves the loss of the 120 kb plasmid 'c' from DH209 (compare Figure 3.3.4, Lane # 9 with Figure 3.3.9, Lane # 1).

The range of plasmid profiles observed for all isolated exconjugants are summarised diagrammatically in Figure 3.3.10. Using this diagram, the exconjugants can be placed into three distinct classes - Class I to Class III - solely on the basis of their plasmid profile. The reason for doing this was to determine any discernable plasmid patterns amongst the exconjugants.

Exconjugants of Class I form the most numerous class, consisting of a total of 23 exconjugants from both crosses. The Class I exconjugants all contain five plasmids, which are ~1000 kb, 570 kb, 295 kb, 163 kb, and 40 kb in size. The Class II make up the next most predominant class, consisting of 14 exconjugants from both crosses. Again, the Class II exconjugants contain five plasmids, but are ~1000 kb, 570 kb, 295 kb, 163 kb, and 22 kb in size. The Class III exconjugants, by default, include all those which do not fit into the two previous classes. The class is made up of 16 exconjugants, each with a unique plasmid profile.

3.3.2 Examination of Variability in CCC Band Size.

A comparison of the plasmid profiles reveals that no isolated exconjugant displays the plasmid profile exhibited by the parental strain, OR168. However, when taken individually, most of the plasmid bands making up the OR168 profile are also represented in each of the exconjugant profiles.

As mentioned above in Section 3.3.1, the OR168 plasmid band identified as the 570-kb pOR168a appears present and stable in all the exconjugants screened.

The plasmid band identified as the 225-kb pOR168b is not present in any of the exconjugants screened, although a new, slightly larger band is present in 75% (40/53) of exconjugants. In the other 25% (13/53) of exconjugants, the plasmid appears over a range of sizes or is missing. The assumption that these plasmids have derived from pOR168b is purely conjecture and is based on plasmids corresponding in size to the other native OR168 plasmids being present in the exconjugants.

The OR168 plasmid band identified as the 163-kb pOR168c appears relatively stable in the exconjugants, with only 3 (5.7%) showing variations in its position. These are DH116, DH122, and DH211 with band sizes corresponding to 120 kb, 200 kb, and 120 kb, respectively.

By comparison, the OR168 band identified as the 22-kb pOR168d is comparatively unstable with only about 40% (21/53) of exconjugants showing a band of this size.

Those exconjugants not having a plasmid band analogous to pOR168d exhibit a new band of 40 kb in all but 3 exconjugants. The coincidental disappearance of the 22-kb band with the appearance of the new 40 kb band suggests the new band has resulted from the 22-kb plasmid undergoing some sort of rearrangement. The three exceptions (DH120, DH125 and DH216) show unique plasmid patterns, with neither a 22-kb nor a 40 kb band. Instead, these three exconjugants show bands of 28 kb, 36 kb, and 65 kb, respectively.

The limitations inherent in measuring distances on gel negatives and photographs will undoubtedly cause variations in the estimated plasmid size. Repeating the Eckhardt gels for the 27 exconjugants used in Section 3.7, and sizing the plasmids visible on them, gave very close agreement to the sizes estimated here. But it must be remembered the plasmid sizes are only estimates and dependent on the accuracies of interpreting plasmid sizing gels like the one shown in Figure 3.2.1.

The plasmid profiles exhibited by the exconjugants for both crosses can be grouped into three classes. The first, most predominant class (Class I) consists of exconjugants showing plasmid bands of 570, 295, 163, and 40 kb. 44% (12/27) of exconjugants from Cross 1 and 42% (11/26) of exconjugants from Cross 2 show this plasmid profile. Another 26% (7/27) of exconjugants from Cross 1 and 27% (7/26) of exconjugants from Cross 2 show plasmid bands of 570, 295, 163, and 22 kb. These exconjugants constitute the second most predominant class. The third class is made up of the remaining 16 exconjugants (8 from each Cross) that do not fit into either the first or second class. Each exconjugant of this class exhibits a unique plasmid profile. A summary of these three classes of plasmid profile patterns for each cross is shown in Figure 3.3.10. Also listed is the frequency at which each pattern occurred and the profile class to which they belong.

The exconjugants displaying the two predominant classes of plasmid patterns common to each cross make up 70% of the isolates screened. The other 30% of exconjugants show a wide range of different profiles, with each profile unique to that particular exconjugant.

There is a possibility that the exconjugants from each cross showing the same plasmid profile are clonal relatives, that is, they have arisen from the same conjugation/transposition event. If this is so, random selection of cross progeny should depict a random subpopulation of the resultant exconjugant population. It is curious then, that 70% (37/53) of the exconjugants from both crosses show one of two types of plasmid profile.

Comparing the plasmid profiles has revealed the high incidence of particular patterns of rearrangement. This suggests the presence of hot-spots for the insertion of Tn*5*. No definitive conclusions can be made about what has caused the observed rearrangements to the plasmid profiles using information gained from the plasmid sizing and comparative profiling exercises, as discussed in this section and Section 3.2. It has given some indications on the direction the investigations should go. The actual sites of Tn*5* insertion and the number of insertion events per genome must be determined to help trace the involvement of the transposon in the resultant rearrangements observed for the exconjugant plasmid profiles.

3.3.3 The Megaplasmid Band

Comparison of the plasmid profiles shows that the weakly staining band with an RM value of 7.0 is not always present for each exconjugant. For example, the plasmid extracts of DH201, DH202 and DH203 shown in lanes 1,2 and 3 respectively of Figure 3.3.4a, do not contain a 7.0 RM band. This may be a direct result of the amount of DNA sample loaded into the well of the Eckhardt gel. Alternatively, it may reflect the treatment of the cells during *in situ* lysis during sample preparation.

Extrapolation of the semi-log plot of plasmid size against RM shown in Figure 3.3.11 gives the 7.0 RM band a size in excess of 1000 kb, which means that if it is plasmid DNA, it would be of the proportions of a megaplasmid. Because of the inability to extrapolate from Figure 3.3.11 with precision, the exact size of the megaplasmid cannot be made. The presence of such megaplasmids in *Rhizobium* species is not uncommon, although even with their low-copy number status, they still amount to a sizable mass of DNA. If the 7.0 RM band is in fact a megaplasmid, with one copy per cell, it should fluoresce more brightly with the ethidium bromide stain. But, as mentioned previously, this may be the result of the megaplasmid often being lost by degradation during sample preparation when setting up the Eckhardt gels. Any nicking or sharing of the megaplasmid DNA, and any resultant conformational changes from this, may inhibit its migration under the electrophoresis conditions used. Such altered megaplasmid DNA may simply be trapped in the agarose forming the wells of the gel.

There also appears to be some variation in the size of the megaplasmid bands visible for two exconjugants. In the initial Eckhardt gels, both DH106 and DH118 appear to have more than one visible band above the 570 kb band (refer to Figs 3.3.1a, lane # 6; and 3.3.3a, lane # 3, respectively). For DH118, this phenomenon does not appear to be consistent (compare Figs 3.3.3a, lane # 3, and 3.3.7a, lane # 9); whilst for DH106 it is (compare Figs 3.3.1a, lane # 6, and 3.3.7a, lane # 2). The inconsistencies with plasmid mobility observed in DH118 suggest that it is the result of a change in the conformation of one plasmid. For DH106, it appears the megaplasmid has a slightly faster mobility, equating to an estimated size of 890 kb. The other replicon visible above the 570 kb band may be a derivative of the original pOR168b plasmid, which appears to be missing from this exconjugant.

Evidence for the 7.0 RM band actually being a unique episome is strengthened by the Eckhardt gel and Southern blot results seen in Figures 3.3.1 and 3.3.2. Interestingly, both of these gels formed the initial blots probed for Tn*5* sequence (see Section 3.5), with the optimisation of conditions for probe hybridisation and detection still being investigated (see Section 2.12). Fortuitously, perhaps, the exconjugants

DH109 and DH123 (Fig 3.3.1b, lane # 9 and Fig 3.3.2b, lane # 5, respectively) show one faint band hybridising with the labelled Tn*5* probe. The detected band corresponds to the weakly staining 7.0 RM band. No other bands containing Tn*5* are visible in either of these samples, although nuclear material trapped in the well has also been detected. If, under the hybridisation conditions used, the detected 7.5 RM band simply represented a plasmid of altered conformation (such as nicked or relaxed DNA), other bands corresponding to more tightly coiled forms would also be expected to be detected (as seen for the exconjugants with plasmid-borne Tn*5* elements on the same blots).

Repeating the Tn*5* probing on DH109 under more stringent hybridisation conditions and using less labelled probe meant that Tn*5* insertion in the megaplasmid was not detected (compare Figures 3.3.1 and 3.3.7). The alteration to the hybridisation conditions used for the Tn*5* probing also reduced the 'laddering' effect. Because of the increased clarity, these conditions were adopted for all subsequent Tn*5* probing. There is also the possibility that under the subsequent hybridisation conditions used, some Tn*5* insertions into the megaplasmid may not have been detected.

Figure 3.3.1: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.1a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.1b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.1a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #	1	DH101*
	2	DH102*
	3	DH103*
	4	DH104*
	5	DH105*
	6	DH106*
	7	DH107*
	8	DH108*
	9	DH109
	10	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.1a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

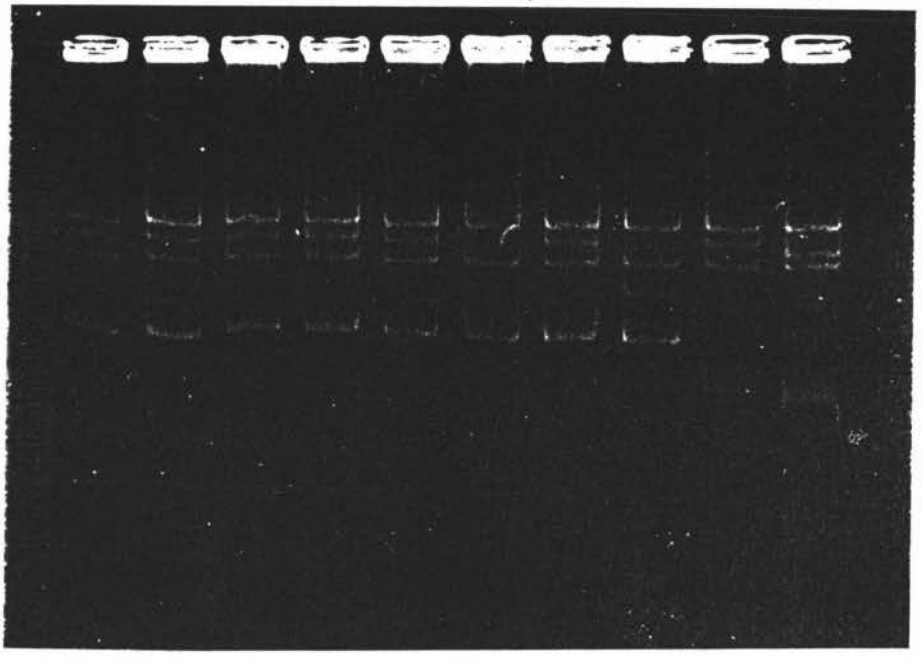


Figure 3.3.1b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

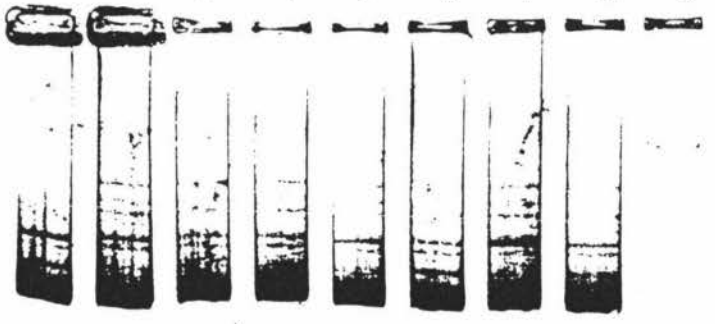


Figure 3.3.2: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.2a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.2b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.2a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #	1	DH110
	2	DH111*
	3	DH112
	4	DH122*
	5	DH123
	6	DH124*
	7	DH125*
	8	DH210
	9	DH211
	10	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.2a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

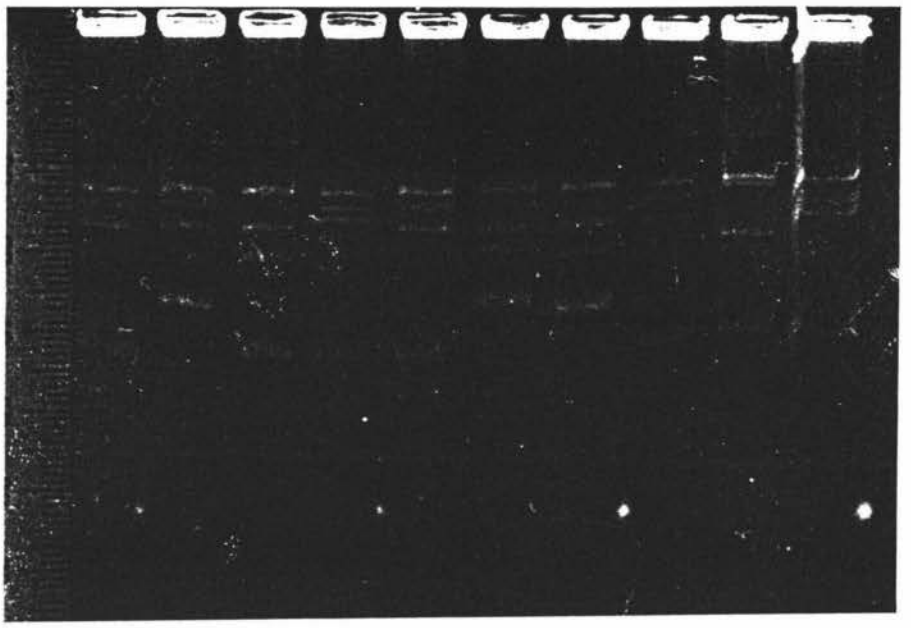


Figure 3.3.2b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

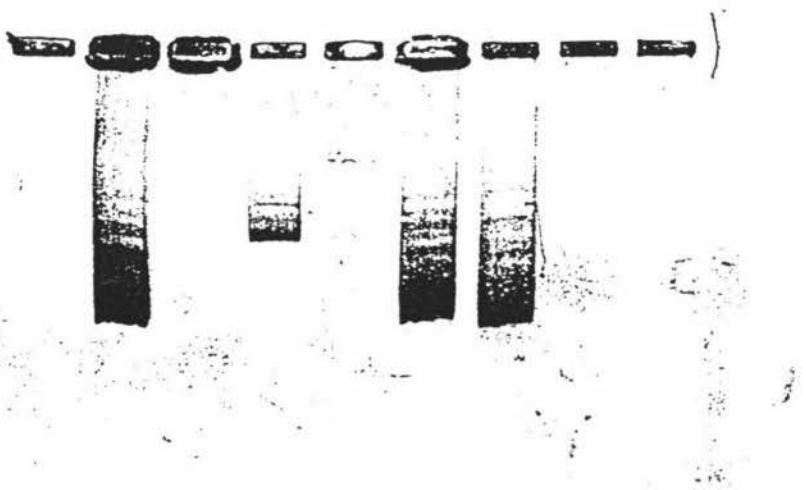


Figure 3.3.3: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.3a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.3b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.3a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #	1	DH120*
	2	DH119*
	3	DH118
	4	DH117
	5	DH116*
	6	DH115
	7	DH114*
	8	DH113*
	9	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.3a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9

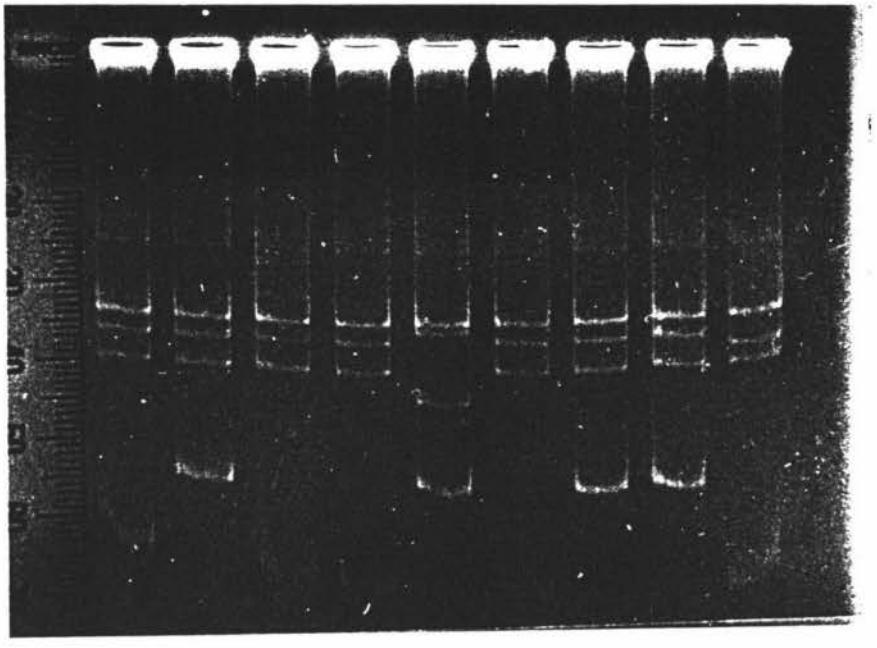


Figure 3.3.3b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9

Figure 3.3.4: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.4a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.4b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.4a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #	1	DH201*
	2	DH202
	3	DH203
	4	DH204
	5	DH205*
	6	DH206*
	7	DH207
	8	DH208
	9	DH209
	10	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.4a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

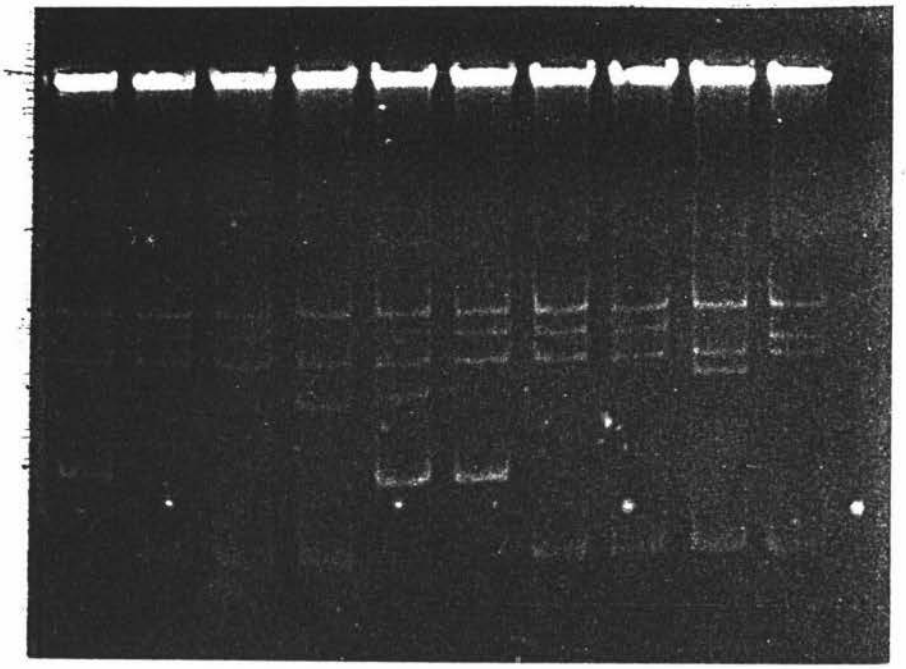


Figure 3.3.4b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

Figure 3.3.5: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.5a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.5b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.5a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #		
	1	DH217*
	2	DH216*
	3	DH215*
	4	DH214
	5	DH213*
	6	DH212*
	7	DH127*
	8	DH126*
	9	DH121*
	10	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.5a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

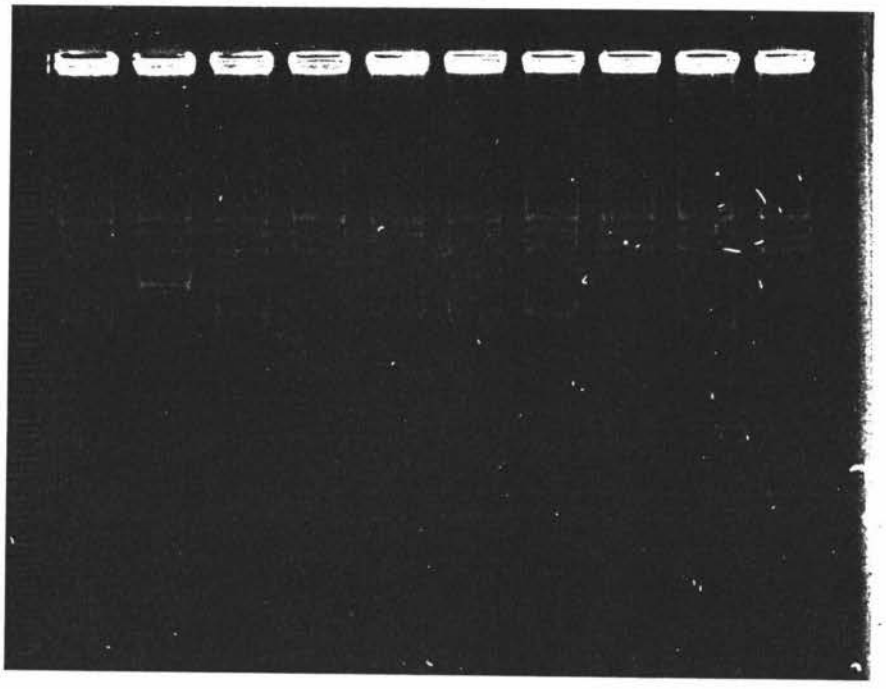


Figure 3.3.5b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

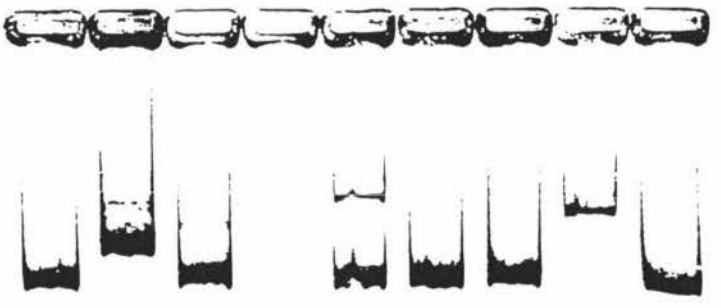


Figure 3.3.6: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.6a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.6b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.6a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #	1	OR168
	2	DH226*
	3	DH225*
	4	DH224*
	5	DH223*
	6	DH222
	7	DH221
	8	DH220*
	9	DH219*
	10	DH218*

ote: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.6a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

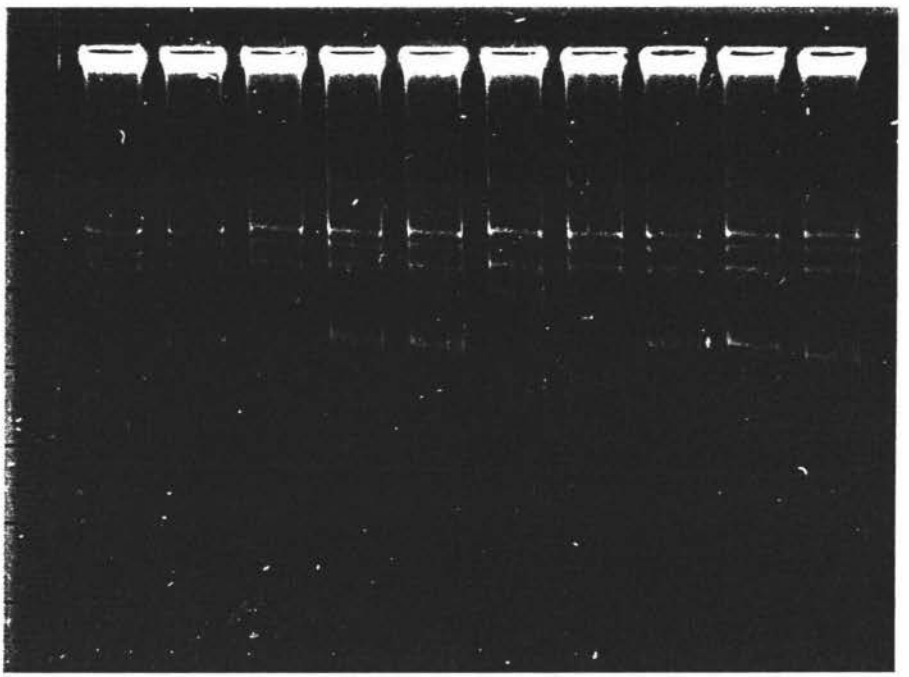


Figure 3.3.6b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

Figure 3.3.7: Plasmid profiles and location of Tn*5* and the RP4-*mob* fragment in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.7a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.7b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.7a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section, using the chromogenic detection system.

Lane #		
	1	DH101*
	2	DH106*
	3	DH108*
	4	DH109
	5	DH112
	6	DH113*
	7	DH115
	8	DH116*
	9	DH118
	10	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.7a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

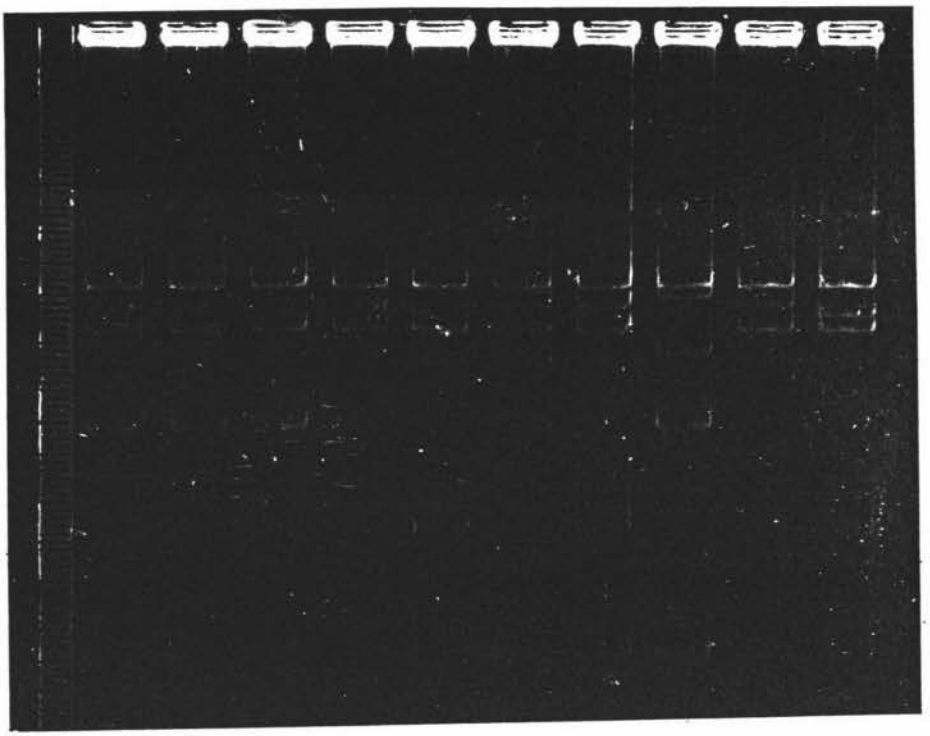


Figure 3.3.7b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

Figure 3.3.7 (Continued): Plasmid profiles and location of Tn*5* and the RP4-*mob* fragment in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.7c. Southern blot of a replica Eckhardt gel of the one shown in Fig. 3.3.7a probed with the DIG-labelled internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps was performed as described in the Materials and Methods section, using a chemiluminescent detection system for simple and rapid removal of all adherent probe sequence, allowing successful reprobing.

Figure 3.3.7d. Southern blot of a replica Eckhardt gel of the one shown in Fig. 3.3.7a, stripped and reprobed with DIG labelled 3.1-kb *Eco*RI/*Hin*dIII fragment of pSUP202 to detect the replicon location of the RP4-*mob* fragment from pSUP1011. The stripping, rehybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #		
	1	DH101*
	2	DH106*
	3	DH108*
	4	DH109
	5	DH112
	6	DH113*
	7	DH115
	8	DH116*
	9	DH118
	10	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.7c: Southern blot of a replica gel of the one shown in Fig. 3.3.7a probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

Figure 3.3.7d: Southern blot of the replica gel stripped and reprobed for RP4-*mob*

Lane #: 1 2 3 4 5 6 7 8 9 10

Figure 3.3.8: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.8a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.8b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.8a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #	1	DH119*
	2	DH120*
	3	DH122*
	4	DH124*
	5	DH125*
	6	DH126*
	7	DH201*
	8	DH204
	9	DH205*
	10	OR168

Note: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.8a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

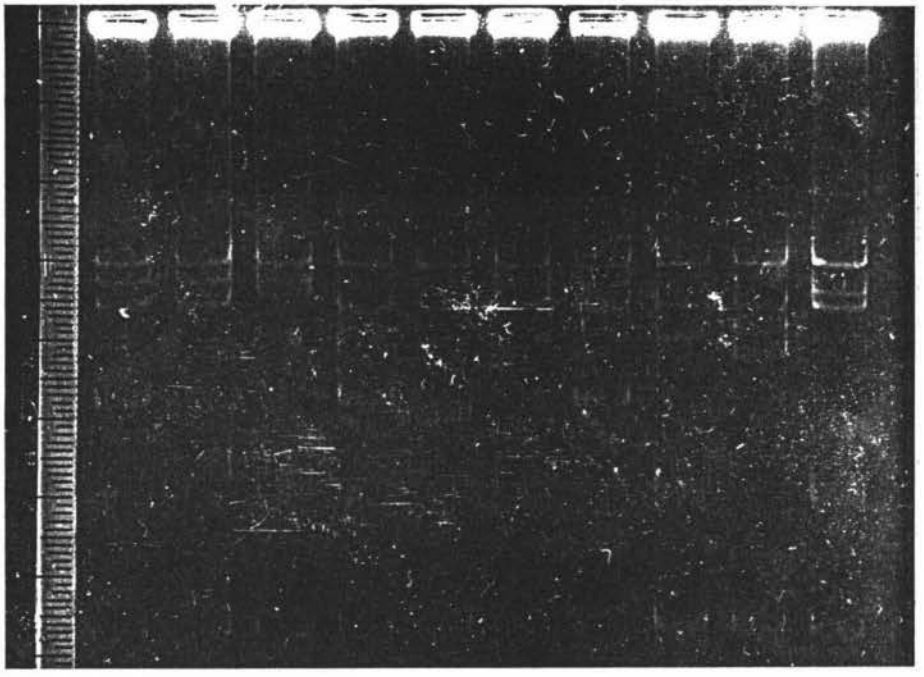


Figure 3.3.8b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

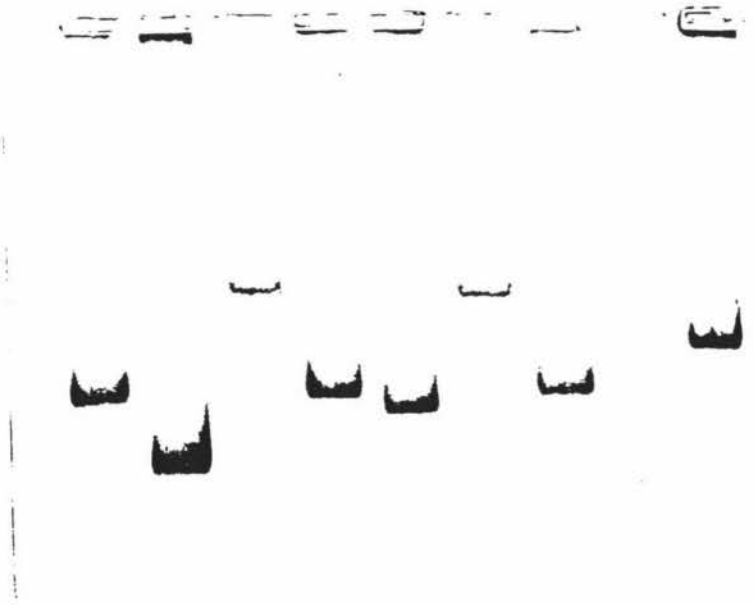


Figure 3.3.9: Plasmid profiles and location of Tn*5* in OR168::Tn*5* exconjugants obtained via heterogeneric conjugation between OR168 and *E. coli* PN302.

Figure 3.3.9a. Eckhardt gel electrophoresis of plasmids isolated from OR168::Tn*5* exconjugants stained with ethidium bromide and compared to the wild-type parental recipient, OR168. Cell lysis, plasmid electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.3.9b. Southern blot of the Eckhardt gel shown above, in Fig. 3.3.9a, probed with the internal *Hin*dIII fragment from pKan2 to detect the replicon location of the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

Lane #		
	1	DH209
	2	DH211
	3	DH212*
	4	DH213*
	5	DH214
	6	DH216*
	7	DH221
	8	DH222
	9	DH225*
	10	OR168

ote: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.3.9a: Eckhardt gel electrophoresis showing plasmid profiles

Lane #: 1 2 3 4 5 6 7 8 9 10

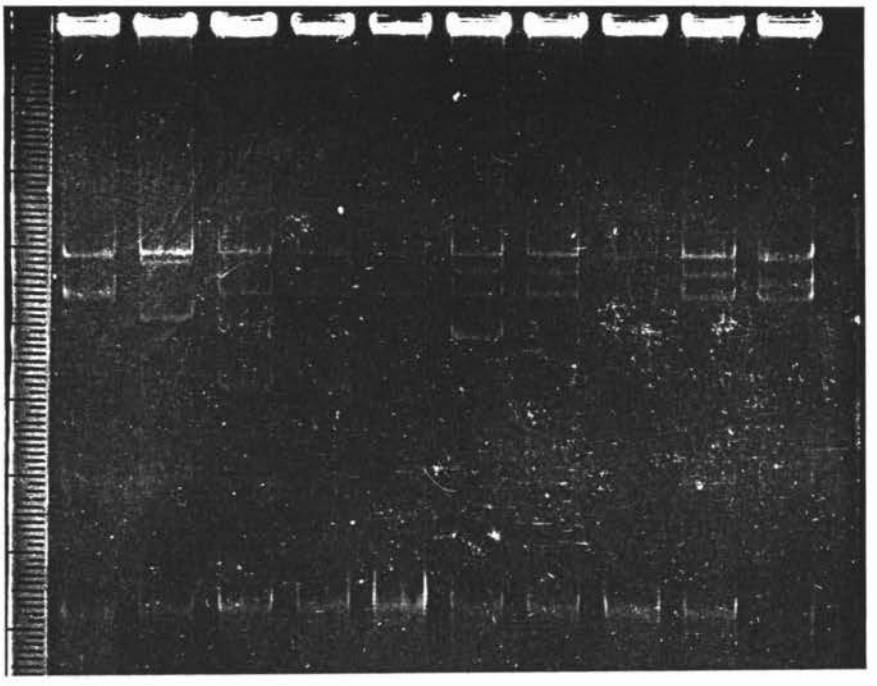


Figure 3.3.9b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10

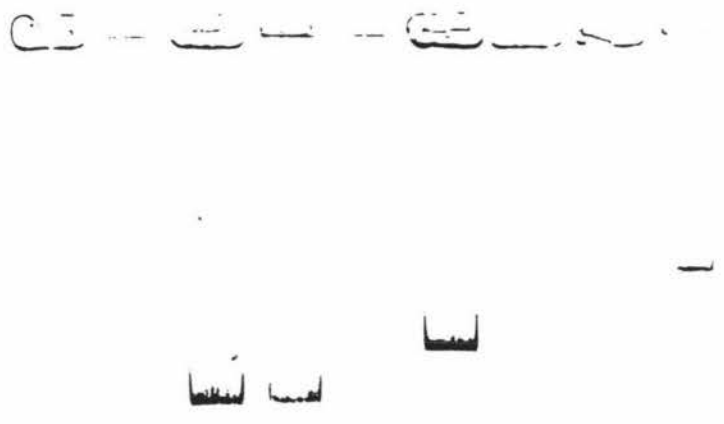
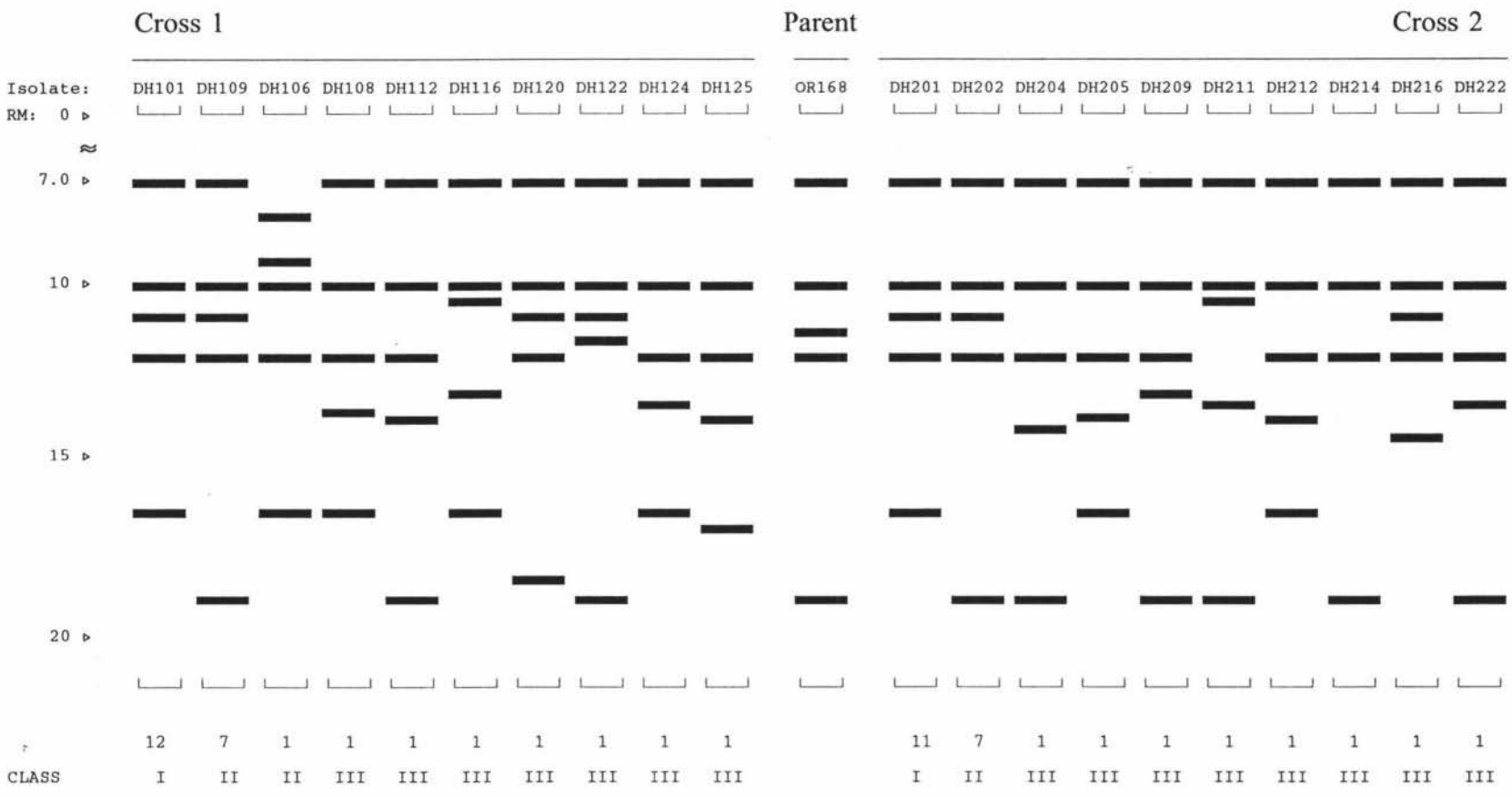
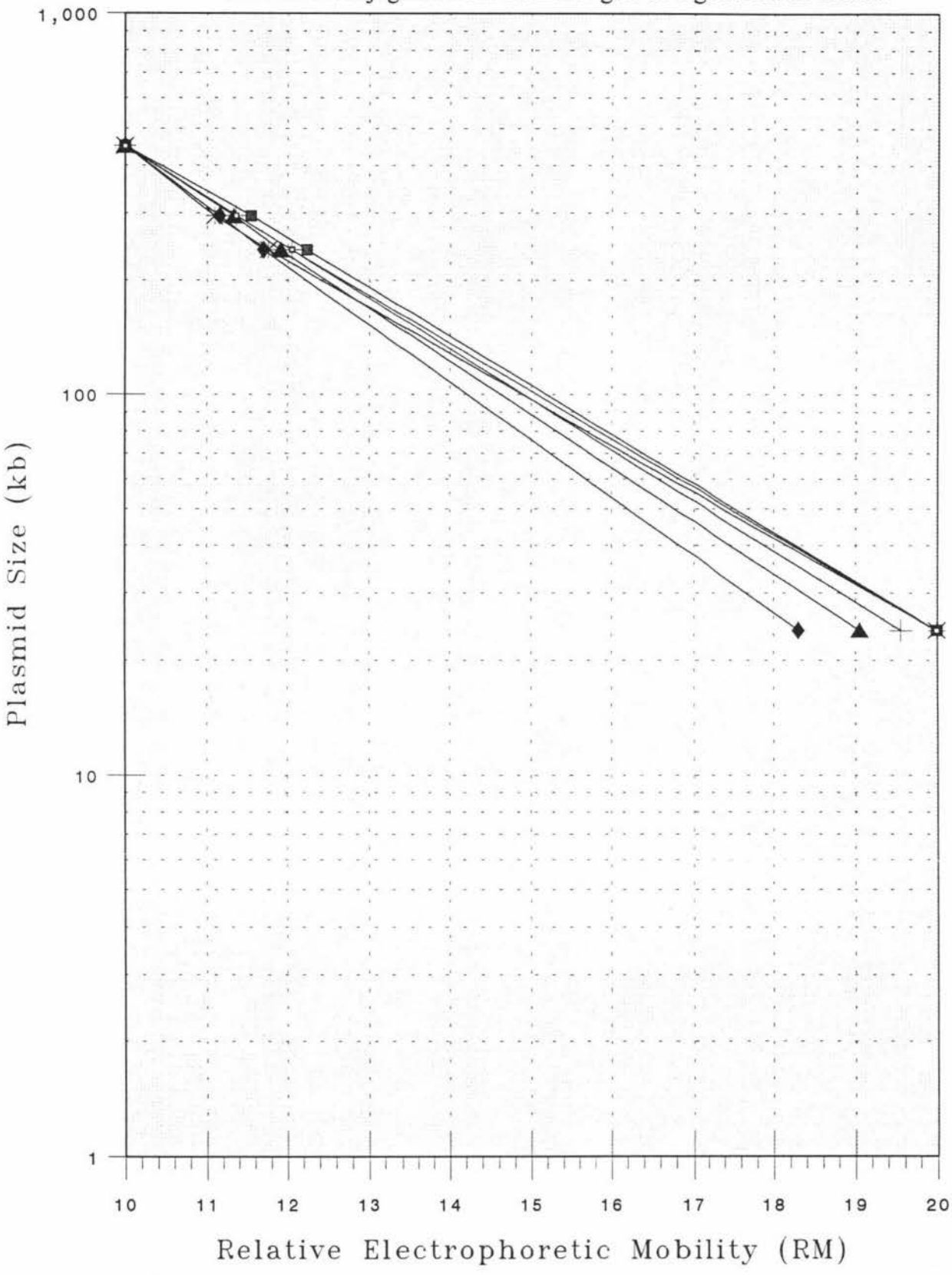


Figure 3.3.10: Summary of the comparative plasmid profiles seen after the conjugative transfer of Tn*5* into OR168



NOTE: The isolate named at the top of each lane indicates the actual plasmid profile shown.
The figures below each lane indicate the number of isolates from the cross with that plasmid profile.
Data summary from gel profiles shown in Figures 3.3.1 to 3.3.9

Figure 3.3.11: Semi-$\log_{10}$ plot of plasmid size against RM for determining plasmid sizes of exconjugants from Eckhardt gels of figs 3.3.1a to 3.3.6a.



Note: Data obtained from gels shown in Figures 3.3.1 to 3.3.6

TABLE 3.3.1: Sizing of plasmid bands visible on gel shown in Figure 3.3.1a.

Lane #	1			2			3			4			5			6			7			8			9			10		
Isolate	DH101			DH102			DH103			DH104			DH105			DH106			DH107			DH108			DH109			OR168		
# BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1 'm'	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	17.0	7.73	~850	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000
2 'a'	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570
3 'b'	24.0	10.91	295	24.0	10.91	295	24.0	10.91	295	24.0	10.91	295	24.0	10.91	295	20.5	9.32	600	24.0	10.91	295	26.5	12.05	163	24.0	10.91	295	25.0	11.36	225
4 'c'	26.5	12.05	163	26.5	12.05	163	26.5	12.05	163	26.5	12.05	163	26.5	12.05	163	26.5	12.05	163	26.5	12.05	163	30.0	13.64	94	26.5	12.05	163	26.5	12.05	163
5 'd'	***36.5***	***16.59***	***40***	***36.5***	***16.59***	***40***	***36.5***	***16.59***	***40***	***36.5***	***16.59***	***40***	***36.5***	***16.59***	***40***	***36.5***	***16.59***	***40***	***36.5***	***16.59***	***40***	***36.5***	***16.59***	***40***	43.0	19.55	22	43.0	19.55	22
CLASS	I			I			I			I			I			III			I			III			II					

TABLE 3.3.2: Sizing of plasmid bands visible on gel shown in Figure 3.3.2a.

Lane #	1			2			3			4			5			6			7			8			9			10		
Isolate	DH110			DH111			DH112			DH122			DH123			DH124			DH125			DH210			DH211			OR168		
# BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1 'm'	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000	15.4	7.00	~1000
2 'a'	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570	22.0	10.00	570
3 'b'	24.0	10.91	295	24.0	10.91	295	26.5	12.05	163	24.0	10.91	295	24.0	10.91	295	26.5	12.05	163	26.5	12.05	163	24.0	10.91	295	23.0	10.45	395	25.0	11.36	225
4 'c'	26.5	12.05	163	26.5	12.05	163	30.5	13.86	86	***25.5***	***11.59***	***200***	26.5	12.05	163	29.5	13.41	120	30.5	13.86	86	26.5	12.05	163	29.5	13.41	120	26.5	12.05	163
5 'd'	42.0	19.09	22	***36.5***	***16.59***	***40***	42.0	19.09	22	42.0	19.09	22	42.0	19.09	22	***36.5***	***16.59***	***40***	***37.5***	***17.05***	***36***	42.0	19.09	22	42.0	19.09	22	42.0	19.09	22
CLASS	II			I			III			III			II			III			III			II			III					

Note: In the tables above, highlighted figures in italics indicate the plasmid of the exconjugant containing Tn*5*. **DM**: distance (mm) band migrated on gel. **RM**: Relative electrophoretic mobility. CLASS: Classification of plasmid profile.

TABLE 3.3.3: Sizing of plasmid bands visible on gel shown in Figure 3.3.3a.

Lane #	1			2			3			4			5			6			7			8			9		
Isolate	DH120			DH119			DH118			DH117			DH116			DH115			DH114			DH113			OR168		
# BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1 'm'	24.0	7.00	~1000	24.0	7.00	~1000	24.0	7.00	~1000	24.0	7.00	~1000	24.0	7.00	~1000	24.0	7.00	~1000	24.0	7.00	~1000	24.0	7.00	~1000	24.0	7.00	~1000
2 'a'	34.0	10.00	570	34.0	10.00	570	34.0	10.00	570	34.0	10.00	570	34.0	10.00	570	34.0	10.00	570	34.0	10.00	570	34.0	10.00	570	34.0	10.00	570
3 'b'	36.3	10.68	295	36.3	10.68	295	36.3	10.68	295	36.3	10.68	295	35.5	10.44	395	36.3	10.68	295	36.3	10.68	295	36.3	10.68	295	37.7	11.09	225
4 'c'	40.0	11.76	163	40.0	11.76	163	40.0	11.76	163	26.5	12.05	163	44.5	13.09	100	40.0	11.76	163	40.0	11.76	163	40.0	11.76	163	40.0	11.76	163
5 'd'	***63.0***	***18.53***	***28***	***55.0***	***16.18***	***40***	68.0	20.00	22	68.0	20.00	22	***55.0***	***16.18***	***40***	68.0	20.00	22	***55.0***	***16.18***	***40***	***55.0***	***16.18***	***40***	68.0	20.00	22
CLASS	III			I			II			II			III			II			I			I					

TABLE 3.3.4: Sizing of plasmid bands visible on gel shown in Figure 3.3.4a.

Lane #	1			2			3			4			5			6			7			8			9			10		
Isolate	DH201			DH202			DH203			DH204			DH205			DH206			DH207			DH208			DH209			OR168		
# BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1 'm'	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000
2 'a'	29.0	10.00	570	29.0	10.00	570	32.0	11.03	295	29.0	10.00	570	29.0	10.00	570	29.0	10.00	570	29.0	10.00	570	29.0	10.00	570	29.0	10.00	570	29.0	10.00	570
3 'b'	32.0	11.03	295	32.0	11.03	295	29.0	10.00	570	35.5	12.24	163	35.5	12.24	163	32.0	11.03	295	32.0	11.03	295	32.0	11.03	295	35.5	12.24	163	33.5	11.55	225
4 'c'	35.5	12.24	163	35.5	12.24	163	35.5	12.24	163	41.0	14.14	83	40.0	13.79	92	35.5	12.24	163	35.5	12.24	163	35.5	12.24	163	37.7	13.10	120	35.5	12.24	163
5 'd'	***50.0***	***17.24***	***40***	60.0	20.69	22	60.0	20.69	22	60.0	20.69	22	***50.0***	***17.24***	***40***	***50.0***	***17.24***	***40***	60.0	19.05	22	60.0	19.05	22	60.0	19.05	22	60.0	19.05	22
CLASS	I			II			II			III			III			I			II			II			III					

Note: In the tables above, highlighted figures in italics indicate the plasmid of the exconjugant containing Tn*5*. **DM**: distance (mm) band migrated on gel. **RM**: Relative electrophoretic mobility. CLASS: Classification of plasmid profile.

TABLE 3.3.5: Sizing of plasmid bands visible on gel shown in Figure 3.3.5a.

Lane #	1			2			3			4			5			6			7			8			9			10		
Isolate	DH217			DH216			DH215			DH214			DH213			DH212			DH127			DH126			DH121			OR168		
# BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1 'm'	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000	14.5	7.00	~1000
2 'a'	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570	20.5	10.00	570
3 'b'	22.5	10.98	295	22.5	10.98	295	22.5	10.98	295	24.7	12.05	163	22.5	10.98	295	24.7	12.05	163	22.5	10.98	295	22.5	10.98	295	22.5	10.98	295	23.5	11.46	225
4 'c'	24.7	12.05	163	24.7	12.05	163	24.7	12.05	163	40.0	19.51	22	24.7	12.05	163	28.5	13.90	86	24.7	12.05	163	***24.7***	***12.05***	***163***	24.7	12.05	163	24.7	12.05	163
5 'd'	33.0	16.10	40	***29.5***	***14.39***	***65***	***33.0***	***16.10***	***40***	-			***33.0***	***16.10***	***40***	***33.0***	***16.10***	***40***	***33.0***	***16.10***	***40***	40.0	19.51	22	***33.0***	***16.10***	***40***	40.0	19.51	22
CLASS	I			III			I			III			I			III			I			II			I					

TABLE 3.3.6: Sizing of plasmid bands visible on gel shown in Figure 3.3.6a.

Lane #	1			2			3			4			5			6			7			8			9			10		
Isolate	OR168			DH226			DH225			DH224			DH223			DH222			DH221			DH220			DH219			DH218		
# BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1 'm'	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000	16.5	7.00	~1000
2 'a'	23.5	10.00	570	23.5	10.00	570	***23.5***	***10.00***	***570***	23.5	10.00	570	23.5	10.00	570	23.5	10.00	570	23.5	10.00	570	23.5	10.00	570	23.5	10.00	570	23.5	10.00	570
3 'b'	26.2	11.15	225	25.3	10.77	295	25.3	10.77	295	25.3	10.77	295	25.3	10.77	295	27.5	11.70	163	25.3	10.77	295	25.3	10.77	295	25.3	10.77	295	25.3	10.77	295
4 'c'	27.5	11.70	163	27.5	11.70	163	27.5	11.70	163	27.5	11.70	163	27.5	11.70	163	31.3	13.32	100	27.5	11.70	163	27.5	11.70	163	27.5	11.70	163	27.5	11.70	163
5 'd'	45.0	19.15	22	***37.5***	***15.96***	***40***	45.0	19.15	22	***37.5***	***15.96***	***40***	***37.5***	***15.96***	***40***	45.0	19.15	22	45.0	19.15	22	***37.5***	***15.96***	***40***	***37.5***	***15.96***	***40***	***37.5***	***15.96***	***40***
CLASS				I			II			I			I			III			II			I			I			I		

Note: In the tables above, highlighted figures in italics indicate the plasmid of the exconjugant containing Tn*5*. **DM**: distance (mm) band migrated on gel. **RM**: Relative electrophoretic mobility. CLASS: Classification of plasmid profile.

TABLE 3.3.7: Sizing of plasmid bands visible on gel shown in Figure 3.3.7a.

Lane #		1			2			3			4			5			6			7			8			9			10		
Isolate		DH101			DH106			DH108			DH109			DH112			DH113			DH115			DH116			DH118			OR168		
#	BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1	'm'	22.5	7.00	~1000	24.5	7.54	~850	22.5	7.00	~1000	22.5	7.00	~1000	22.5	7.00	~1000	22.5	7.00	~1000	22.5	7.00	~1000	22.5	7.00	~1000	22.5	7.00	~1000	22.5	7.00	~1000
2	'a'	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570	32.5	10.00	570
3	'b'	34.5	10.62	295	29.0	9.20	600	38.0	11.69	163	34.5	10.62	295	38.0	11.69	163	34.5	10.62	295	34.5	10.62	295	33.5	10.31	395	34.5	10.62	295	36.0	11.08	225
4	'c'	38.0	11.69	163	38.0	11.69	163	42.0	12.92	100	38.0	11.69	163	42.0	12.92	100	38.0	11.69	163	38.0	11.69	163	41.0	12.62	120	38.0	11.69	163	38.0	11.69	163
5	'd'	***51.0***	***15.69***	***40***	***51.0***	***15.69***	***40***	***51.0***	***15.69***	***40***	63.5	19.54	22	63.5	19.54	22	***51.0***	***15.69***	***40***	63.5	19.54	22	***51.0***	***15.69***	***40***	63.5	19.54	22	63.5	19.54	22
CLASS		I			III			III			II			III			I			II			III			II					

TABLE 3.3.8: Sizing of plasmid bands visible on gel seen in Figure 3.3.8a.

Lane #		1			2			3			4			5			6			7			8			9			10		
Isolate		DH119			DH120			DH122			DH124			DH125			DH126			DH201			DH204			DH205			OR168		
#	BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1	'm'	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000	20.5	7.00	~1000
2	'a'	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570	29.5	10.00	570
3	'b'	32.5	11.02	295	32.5	11.02	295	32.5	11.02	295	35.0	11.86	163	35.0	11.86	163	32.5	11.02	295	32.5	11.02	295	35.0	11.86	163	35.0	11.86	163	33.5	11.36	225
4	'c'	35.5	12.24	163	35.5	12.24	163	***34.0***	***11.53***	***200***	38.0	12.88	106	39.0	13.22	97	***35.0***	***11.86***	***163***	35.0	11.86	163	39.5	13.39	92	38.5	13.05	102	35.0	11.86	163
5	'd'	***47.5***	***16.10***	***51***	***56.0***	***18.98***	***28***	59.5	20.17	22	***47.5***	***16.10***	***51***	***49.5***	***16.78***	***44***	59.5	20.17	22	***47.5***	***16.10***	***51***	59.5	20.17	22	***41.5***	***14.07***	***78***	59.5	20.17	22
CLASS		I			III			III			III			III			II			I			III			III					

Note: In the tables above, highlighted figures in italics indicate the plasmid of the exconjugant containing Tn*5*. **DM**: distance (mm) band migrated on gel. **RM**: Relative electrophoretic mobility. CLASS: Classification of plasmid profile.

TABLE 3.3.9: Sizing of plasmid bands visible on gel seen in Figure 3.3.9a.

Lane #		1			2			3			4			5			6			7			8			9			10		
Isolate		DH209			DH211			DH212			DH213			DH214			DH216			DH221			DH222			DH225			OR168		
#	BAND	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb	DM	RM	kb
1	'm'	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000	21.0	7.00	~1000
2	'a'	30.0	10.00	570	30.0	10.00	570	30.0	10.00	570	30.0	10.00	570	30.0	10.00	570	30.0	10.00	570	30.0	10.00	570	30.0	10.00	570	***30.0***	***10.00***	***570***	30.0	10.00	570
3	'b'	35.5	11.83	163	31.0	10.33	400	35.5	11.83	163	32.5	10.83	295		-		32.5	10.83	295	32.5	10.83	295	35.5	11.83	163	32.5	10.83	295	33.5	11.17	225
4	'c'		-		38.0	12.67	117	39.0	13.00	103	35.5	11.83	163	35.5	11.83	163	35.5	11.83	163	35.5	11.83	163	39.0	13.00	100	35.5	11.83	163	35.5	11.83	163
5	'd'	60.0	20.00	22	60.0	20.00	22	***47.5***	***15.83***	***40***	***47.5***	***15.83***	***40***	60.0	20.00	22	***41.0***	***13.67***	***65***	60.0	20.00	22	60.0	20.00	22	60.0	20.00	22	60.0	20.00	22
CLASS:		III			III			III			I			III			III			II			III			II					

Note: In the table above, highlighted figures in italics indicate the plasmid of the exconjugant containing Tn*5*. **DM**: distance (mm) band migrated on gel. **RM**: Relative electrophoretic mobility. CLASS: Classification of plasmid profile.

3.4 Screening of Exconjugants for Antibiotic Resistance Markers

Before any alterations in the plasmid profiles of the exconjugant population were evident, the assumption had been made that the phenotypic expression of kanamycin resistance (Nm^R) was the result of the acquisition of Tn*5* through a transposition event. Because of the narrow host range of the 12.6 kb mobilisable pSUP1011 vector, its independent persistence after transfer into OR168 was assumed impossible. This now had to be verified along with checking for the possibility of integration of pSUP1011 into the exconjugant genome. This is because Tn*5* can also promote replicon fusion (Berg, 1989).

Phenotypic expression of a plasmid-borne antibiotic resistance marker is indicative of the plasmid bearing that gene being present in that exconjugant isolate. The 'suicide' plasmid pSUP1011 used for the delivery of Tn*5* into OR168 carries the chloramphenicol resistance marker. The presence of a functional Cm^R gene can be determined quickly and easily for the exconjugants using the culture screening methods detailed in Section 2.13. The OR168 parent was shown to be clearly both chloramphenicol-sensitive (Cm^S) and Neomycin-sensitive (Nm^S) using these antibiotic resistance screening methods.

Both the independent persistence or the integration of pSUP1011 in the recipient genome can be monitored using the chloramphenicol resistance marker. These screening methods indicated the loss of pSUP1011: all exconjugants were Sp^R Nm^R Cm^S. The presence of pSUP1011 would leave the exconjugant Cm^R. The absence of pSUP1011 from the exconjugant genome means some other explanation is needed for the profile alterations.

At the outset of this experiment the assumption was made that the chloramphenicol resistance gene, if present, can be expressed in the exconjugant. This has not been independently ascertained. The lack of Cm^R may be the result of the exconjugants being unable to express the gene. So the results would prove the presence of pSUP1011 (or that part of the plasmid coding for chloramphenicol-resistance gene) if the exconjugant was positive for Cm^R, but being negative does not prove its absence.

The presence or absence of pSUP1011 in the exconjugants can be clarified using genotypic methods. This involves probing for DNA sequences specific for the vector. Results for this experiment are detailed in Section 3.6.

3.5 Determining the Role of Tn*5* in the Observed Plasmid Rearrangements

The plasmid complement of OR168 appears to be very stable; after many generations of continuous subculture the same plasmid profile is still seen. An explanation is needed for the cause of the observed alterations in plasmid profiles after the introduction of Tn*5* into OR168. The cause for this may be ascertained by determining the exact position and copy number of Transposon Tn*5* in each exconjugant. A correlation may be found between plasmids with altered mobility and those containing Tn*5*. As well as knowing the sites of Tn*5*-insertions, it will also be very useful to know their precise number in each exconjugant genome. Multiple sites of Tn*5*-insertion may allow plasmid rearrangements through homologous recombination events. These include events such as a single reciprocal crossover resulting in the integration of pSUP1011 into the recipient genome; or more elaborate double crossover events involving integration of only part of the vector. Multiple sites of Tn*5* insertion may also allow the formation of episomal cointegrates.

3.5.1 Determining the Site of Tn*5* Insertion

The site of Tn*5* insertion in the genome of the exconjugants was initially verified using Southern blot hybridisation of the Eckhardt gels shown in Figures 3.3.1a to 3.3.9a using a probe specific for Tn*5* (See Section 2.8). Southern blotting the Eckhardt gels can also determine if the transposon is chromosome-borne or plasmid-borne. If plasmid-borne, it will detect which plasmids contain the transposon.

The blots were hybridised with a Digoxigenin-labelled Tn*5* probe prepared by random-primed labelling of the 3.4-kb *Hin*dIII fragment from pKan2 (See Section 2.11). Specific probe hybridisation was detected using the colour substrates NBT and BCIP, with colour development being allowed to proceed up to 15 hours. The pKan2-hybridisation results of each Eckhardt gel are shown below the gel photograph in Figures 3.3.1b to 3.3.9b.

The hybridisation results for each gel showed a zone corresponding to Tn*5* localized at the well for each exconjugant. This signal has detected the Tn*5* present amongst the chromosomal DNA and large open circular (OC) plasmid DNA that, because of size, could not migrate through the gel. No corresponding signal was detected at the wells that contained DNA from OR168. This verified the lack of sequences

homologous to the Tn*5* probe DNA in the OR168 genome. By extension, this result also verifies the specificity of the probe for Tn*5* sequence only.

By comparing the changes in plasmid mobility with plasmids containing Tn*5*, the results show that change in mobility does not correspond exactly with the inheritance of a Tn*5* element. Although some plasmids carrying Tn*5* have altered mobility, many others have altered mobility without carrying the transposon. The results also suggest non-random insertion of Tn*5* in the genome. This is discussed in more detail in Section 3.5.1.2. A summary of the observed plasmid profiles showing the location of Tn*5*-bearing plasmids is shown in Figure 3.5.1.

3.5.1.1 Multiple Bands

The Tn*5* Southern hybridisation results for the gels of Figures 3.3.1 and 3.3.2 show an unexpected pattern of colour development. In Figure 3.3.1b, for the exconjugants in lanes 1 to 8, there appears to be up to eight bands detected above the 40-kb plasmid band containing Tn*5*. The positions of three of these eight faint bands detected may correspond in mobility to other plasmids visible on the EtBr-stained gel in Figure 3.3.1a. Clearly, the other faint bands do not. The formation of this 'ladder' of bands could be the result of two different phenomena: either the Tn*5*-containing plasmid is present with many different levels of supercoiling; or the effect is caused by non-specific hybridisation, which is usually caused by inadequate stringency during the post-hybridisation washes. There is, of course, no strong argument to support non-specific hybridisation as the cause of the 'laddering', because the lane containing OR168 DNA on both blots is completely clear of any signal.

Alternatively, allowing the colour reaction to proceed for an extended length of time may have permitted the detection of probe sequences at extremely low levels. A similar result is seen in the Southern blot for the exconjugants with plasmid-borne Tn*5* elements in Figure 3.3.2. Primarily, the difference in the level of Tn*5* detection seen in the blots shown in Figs 3.3.1 and 3.3.2, when compared to the other Tn*5*-probed Eckhardt gel blots, is probably caused by the difference in the length of time allowed for the colour reaction to develop during the detection stage. For both Figs 3.3.1 and 3.3.2, colour development was allowed to proceed overnight before the reaction was stopped. For the other blots, colour development only proceeded for a few hours (until strong colour development was seen) before being stopped.

The resulting 'laddering' effect seen with the over-developed blots is interesting in that it suggests that the target plasmid is present in many different conformational forms within the particular exconjugant population. It has only been detected because colour development has been allowed to proceed for an excessive period of time. In most cases, the bands detected on the blot do not equate to any band visible

on the EtBr-stained gel, which is not unusual regarding the high sensitivity of the detection system.

It has also been assumed that the second lighter bands seen in lane 2 (DH 216) and lane 5 (DH213) of Figure 3.3.5b, are the result of the Tn*5*-containing plasmid being present in an altered form, rather than being second sites of Tn*5* insertion. In both cases, the position of the second band corresponds to the position of a visible plasmid band on the gel. But because the relative colour intensity is much weaker than for the relative amount of plasmid DNA present, the assumption has been made that it is due to the presence of co-migrating Tn*5*-bearing plasmid with altered conformation. Again, the causes may be similar to those responsible for the excessive colour development seen in Figs 3.3.1.b and 3.3.2b. The ambiguity created here by the presence of multiple banding may have been eliminated by further post-hybridisation washes using increased stringency, which results in a loss of sensitivity for the detection of homologous DNA sequences. It is probably for this reason that no exconjugants, except DH109 and DH123, showed Tn*5* insertion in the megaplasmid. The combination of low levels of target DNA, a reduced amount of labelled probe DNA being used in the hybridisations, as well as the shorter colour development time could have resulted in Tn*5* not being detected under these conditions.

The assumption that these second lighter bands are not additional sites of Tn*5*-insertion is partially confirmed from results shown in Section 3.5.2. Analyses of the Tn*5*-hybridisation patterns of the *Eco*RI restriction fragments shown in Figure 3.5.2b, show both DH213 (which showed the strongest colour development of the second bands to Tn*5*-probe detection of the Eckhardt gels) and DH216 appear to contain a single Tn*5* element. Also, probing of the Eckhardt gels shown in Figures 3.7.10 to 3.7.12 with the pKan2-derived Tn*5* probe detects a single plasmid band containing Tn*5* for both DH213 and DH216, as well as showing no plasmid-borne Tn*5* element in DH109. Unfortunately, the exconjugants DH123, DH215 and DH226 were not similarly checked again.

Similar hybridisation and detection conditions were used for Tn*5* probing of the total genomic Southern blot shown in Figure 3.5.2b as were used for Figs 3.3.1 and 3.3.2. Again signal intensity for Tn*5* has caused strong colour development in some lanes. This would have been reduced by decreasing the colour development time. The variability in the colour development between lanes is most likely due to variation in the amount of DNA loaded in each lane, which in turn, would vary the amount of target sequence for the probe.

3.5.1.2 Suggestion of Non-Random Insertion of Tn5

The Eckhardt gel band profiles of the sized plasmids in Figure 3.2.1 show an approximate inverse log-linear relationship between plasmid size and plasmid mobility. Using this relationship, the approximate size of plasmids native to OR168, as estimated from the graph in Figure 3.3.11, are as follows: pOR168m, ~1000 kb; pOR168a, 570 kb; pOR168b, 225 kb; pOR168c, 163 kb; and pOR168d, 22 kb. By extrapolating from Figure 3.3.11, insertion of the 5.8 kb Tn*5* element into the 22 kb plasmid is estimated to cause a shift in RM of approximately 0.9 units (approximately 2 to 4 mm). By comparison, the resultant shift in the RM value with Tn*5* insertion into the 570 kb plasmid is too small to be detected by electrophoresis alone. However, Southern blotting of plasmid profile gels shows the exact location of Tn*5*.

It is assumed that due to their large size, the high molecular weight plasmids of OR168 have a low copy number (less than 10 copies per cell). Physical mapping of the *R. meliloti* 1021 genome has shown that it consists of a 3.4 Mb chromosome, plus two pSym megaplasmids of 1.7 Mb and 1.4 Mb (Honeycutt *et al.*, 1993). On the basis of 16S rRNA sequence analysis, OR168 and *R. meliloti* are phylogenetically close (Sivikumaran, PhD thesis, in preparation). Accordingly, it is also assumed that the size of the OR168 chromosome is 3.4 Mb. Thus, making the further assumption that all plasmids native to OR168 are low copy-number replicons, it has been estimated that plasmids make up approximately 20% to 40% of the total genomic DNA in OR168. On this basis, less than 50% of exconjugants are expected to carry Tn*5* on a plasmid, if the site of insertion is truly random and the growth rates of all the exconjugants is approximately equal.

Among the screened exconjugants from Cross 1, approximately 80% (22/27) are shown to contain a plasmid-borne Tn*5* element. Approximately 60% (15/26) of screened Cross 2 exconjugants contained plasmid-borne Tn*5* elements. This corresponds to a much higher frequency of plasmid DNA being involved as target sites for insertion of the transposon than expected.

Closer examination of Cross 1 exconjugants shows 60% (16/27) have a Tn*5* element located in the new 40 kb plasmid. This Tn*5*-bearing plasmid accounts for a high proportion (80% or 16/20) of exconjugants from this cross with a plasmid-borne Tn*5* element. The 12 exconjugants displaying the predominant plasmid pattern seen in this cross (Class I exconjugants) all contain this 40 kb Tn*5*-bearing plasmid. The next most predominant band pattern (Class II exconjugants) in Cross 1 includes 7 of the 27 exconjugants, with only three exconjugants containing a plasmid-borne Tn*5* element. Two of these exceptions contain the Tn*5* in the megaplasmid. The third exception is DH126 with Tn*5* in the 163 kb pDH126c plasmid.

Similarly 50% (13/26) of the exconjugants from Cross 2 have a Tn*5* element in the new 40 kb plasmid. Again this plasmid accounts for a high proportion (87% or 13/15) of exconjugants from this cross with the plasmid-borne Tn*5* element, and include those 11 exconjugants showing the predominant plasmid pattern (Class I) for this cross. The second most common band pattern (Class II) exhibited by exconjugants from Cross 2 (8/26) contain only one example with a Tn*5*-bearing plasmid: DH225 which shows Tn*5* in the 570 kb band.

A total of 16 exconjugants (8 from each Cross) show unique plasmid profiles (Class III), and many of these contain a plasmid-borne Tn*5* element. See Figure 3.3.10 for a summary of the plasmid profiles observed.

In summary 70% (37/53) of exconjugants screened contain a plasmid-borne Tn*5* element and 83% (29/35) of those carry it in the new 40 kb plasmid. Additionally, the acquisition of Tn*5* has involved the small 22-kb pOR168d plasmid in 32 of the 53 events screened. If insertion of Tn*5* into the genome of OR168 is totally random, then one transposition event in every 160 to 170 is expected to involve the pOR168d plasmid. This suggests either host factors or factors peculiar to the transposon delivery system used are strongly influencing the site of insertion of Tn*5* and may also be responsible for the high frequency of rearrangements seen in OR168 exconjugants.

With this stated, it could also be argued that if the small 22 kb plasmid of OR168 is a high copy number replicon, then collectively it could provide a significant mass of target DNA for Tn*5* insertion. The resulting bias may tend to distort the frequency of insertion events involving this plasmid, as observed.

Of the 16 exconjugants showing no plasmid-borne Tn*5* element (5/27 for Cross I; 11/26 for Cross II), 10 have plasmid profiles depicted by Class II exconjugants, while 6 display unique profiles. There is no obvious reason why those exconjugants with chromosomal sites of Tn*5* insertion display an array of different plasmid profiles.

3.5.2 Determining the Number of Tn*5* Elements in Exconjugant Genomes

The Tn*5* hybridisation results of the Eckhardt gels clearly show evidence of plasmid-borne Tn*5* elements. But it is not clear from these blots alone, how many elements are present in the exconjugants. This is for several reasons. For example, for all exconjugants, the DNA trapped in the well of the gel has also hybridised to the Tn*5* probe DNA, even when the probing results indicates the presence of a plasmid-borne element. Does this mean that those exconjugants with plasmid-borne elements carry Tn*5* on their chromosome as well? Also, the laddering effect seen when probing the gels seen in Figures 3.3.1 and 3.3.2 for Tn*5* needs a closer investigation to check if this is due to further sites of Tn*5* insertion in the genome. Another point is that even though these probing results show different replicons containing Tn*5*, the number of elements within the replicon is not revealed.

Clarification of these points can be achieved relatively easily by extracting genomic DNA and digesting with a suitable restriction enzyme that does not cut within Tn*5*. By electrophoretically separating the fragments generated by restriction digestion, and carrying out Southern hybridisation using a labelled Tn*5*-specific probe, the number of Tn*5* elements within each exconjugant can be ascertained.

Isolated total genomic DNA was digested with *Eco*RI. This restriction enzyme has a 6-base recognition sequence with no site within Tn*5*. Because of this, the number of different fragments specifically hybridising to the Tn*5* probe (the internal 3.5-kb *Hin*dIII fragment of pKan2) after Southern blotting should indicate the number of copies of the transposon present, as well as showing the size of the fragment in which it is contained.

It has been assumed exconjugants from the same cross showing the same plasmid profile are clonal sisters, having originated from the same insertion event. With this in mind, 18 exconjugants (9 from each cross) were analysed to determine whether any had multiple copies of Tn*5* in their genome. Each exconjugant tested was chosen on the basis of its plasmid profile with examples depicting most of the patterns summarised in Figure 3.5.1 being analysed.

3.5.2.1 Genomic DNA Restriction Analysis

Using methods detailed in Section 2.10, total genomic DNA was extracted from 18 exconjugants and the parental strain OR168. The genomic DNA was then digested with *Eco*RI and the fragments separated by gel electrophoresis. The gel result is shown in Figure 3.5.2a with the same restriction patterns evident in each lane below the 23.1 kb marker (except for lane # 10 because insufficient DNA was loaded, and lane # 19 because of incomplete digestion). This result is typical of organisms with

the same genomic make-up and is supporting evidence that each exconjugant was derived from OR168. Total genomic restriction patterns have been used for typing bacterial strains with the banding pattern achieved using specific restriction enzymes acting as a "fingerprint".

A Southern blot was made of the gel seen in Figure 3.5.2a and it was probed for Tn*5* sequences with DIG-labelled *Hin*dIII internal fragment of pKan2. This was carried out using the methods detailed in Sections 2.8 and 2.10. Figure 3.5.2b shows the Southern blot analysis of the *Eco*RI restriction digests of genomic DNA from the exconjugants.

The probe hybridisation result indicates a single Tn*5* band for all exconjugants screened, but an unsatisfactory amount of colour development has occurred in many of the lanes. The smearing observed on the blot may have been caused by the combination of some star activity by the *Eco*RI enzyme due to excessive digestion times and the excessive time allowed for the colour development. The reason for carrying out the restriction digests overnight was to avoid any partial digestion products, which would produce multiple banding during the probe detection stage. The resulting background makes interpretation of the blot less clear.

Since Tn*5* contains no restriction sites for *Eco*R1, the number of hybridising fragments on the gel should correspond to the number of Tn*5* elements in the genome of each exconjugant. For the exconjugants screened it appears each contains only a single copy of Tn*5* because there is only a single band detected by the Tn*5* probe. The one exception is lane 19 (DH211): the multiple banding is not unexpected since the DNA appears to be only partially digested for this sample (refer to lane # 19, Fig 3.5.2a and note the smear of DNA fragments).

Further analysis of the Tn*5* probe hybridisation results shown in Figure 3.5.2b revealed that each exconjugant that did not carry a plasmid-borne Tn*5* element shows a single fragment giving a positive hybridisation signal that is in the size range of 7.4 to approximately 23 kb. The wide range of fragment sizes here, corresponds to the random insertion of Tn*5* into the chromosomal DNA of the recipient.

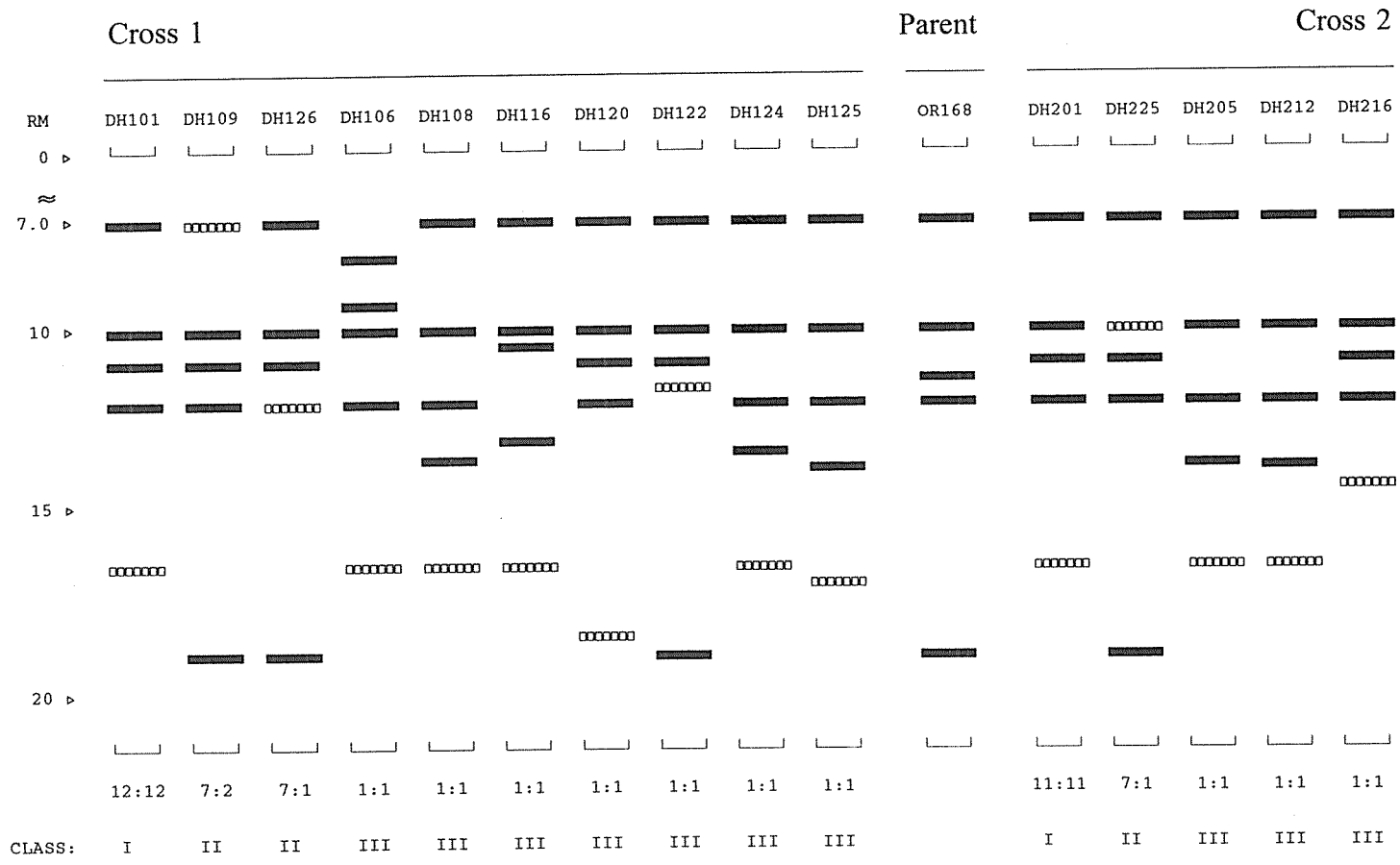
In OR168 there appears to be no strongly staining bands above the 23.1 kb marker. In contrast, for many of the exconjugants screened, bands are visible above the 23.1 kb marker. This variation in the banding pattern coincides with the insertion of Tn*5* into a plasmid site. Exconjugants displaying chromosomal insertion of Tn*5* have a band pattern similar to OR168 above the 23.1 kb marker, namely no visible bands. Without exception, exconjugants with plasmid-borne Tn*5* elements show visible fragments, that are well in excess of 23.1 kb. It appears that it is this band that is

hybridising to the Tn*5* probe. Accurate sizing of the hybridising fragments cannot be done from the gel, but a rough estimate showed the Tn*5* to reside in *Eco*RI fragments that are between 23 kb and 30 kb in size. This curious observation is investigated and discussed in more detail in Section 3.7.

The presence of a single band hybridising to the Tn*5* probe indicates a single site for Tn*5* insertion in the exconjugant genome, regardless of whether the site is chromosomal or plasmidic. This result has eliminated the ambiguity in determining the number of Tn*5* sites that arose from the hybridisation signals seen, either in the 'laddering' effect, or when both a band and the well lit up, in the Eckhardt gel Tn*5* probing results shown in Section 3.3.

Ideally, a Southern blot of *Bam*HI-digested genomic DNA of the same samples should also have been probed with an IS*50*-specific probe to check for evidence of individual IS*50* elements. This is because these terminal inverted repeats of Tn*5* can transpose independently of the Tn*5*. If with the original transfer of Tn*5*, multiple insertions of the transposon had occurred and this was followed by independent IS*50* transposition, it would produce homologous sites at which recombination events could occur. The lack of an IS*50*-specific probe prevented further investigation of this point. With the wisdom of hindsight, the blots could have been probed with pSUP1011 to identify IS*50* sequences. This was done in Section 3.7, when probing for pSUP1011 sequences in the three exconjugants, DH113, DH201, and DH216. None of these three exconjugants showed any evidence of the presence of secondary IS*50* sequences. From this result it can be concluded that rearrangements due to multiple IS*50* elements probably did not occur.

Figure 3.5.1: Summary of comparative plasmid profiles of exconjugants with plasmid-borne Tn*5* elements



NOTE: The figures below each lane express the ratio of the total number of exconjugants from the cross displaying that particular plasmid profile shown, to the number with that profile containing Tn*5* in the plasmid (indicated with ▭). Plasmid bands not containing Tn*5* are shown as solid lines. For some Class II exconjugants, the same plasmid profile is shared by exconjugants with Tn*5* located within different plasmids. A total of 10 Class II exconjugants do not contain a plasmid-borne Tn*5* element. In these the Tn*5* is located in the chromosome.

igure 3.5.2: Electrophoresis and detection of Tn*5* in total genomic DNA from OR168::Tn*5* exconjugants.

igure 3.5.2a. Agarose gel electrophoretic separation of *Eco*RI-digested genomic)NA from selected OR168::Tn*5* exconjugants. The gel was stained with ethidium romide. DNA preparation and digestion, electrophoresis, and gel staining were erformed as described in Materials and Methods section.

igure 3.5.2b. Southern hybridisation of the gel shown above, in Fig. 3.5.2a, probed ith the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the n*5* element. Hybridisation and detection steps were performed as described in the laterials and Methods section.

Lane #		
	1	OR168
	2	DH113*
	3	DH115
	4	DH116*
	5	DH122*
	6	DH124*
	7	DH125*
	8	DH106*
	9	DH108*
	10	DH112
	11	DH212*
	12	DH213*
	13	DH214
	14	DH216*
	15	DH221
	16	DH222
	17	DH205*
	18	DH209
	19	DH211
	20	*Hin*dIII λ DNA size markers

ote: Exconjugants highlighted with an asterisk contain a plasmid-borne Tn*5* element.

Figure 3.5.2a: Gel electrophoresis of *Eco*RI digest fragments of genomic DNA

Lane #: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

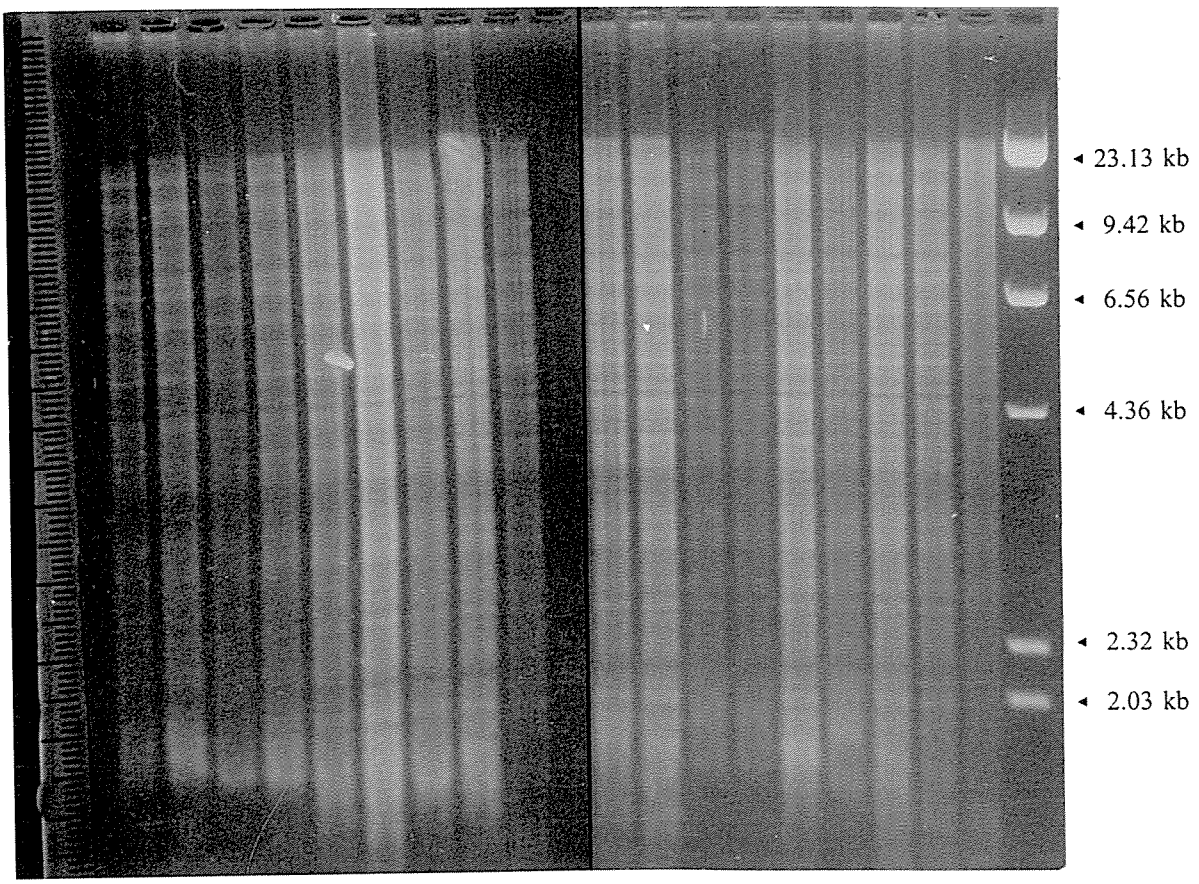
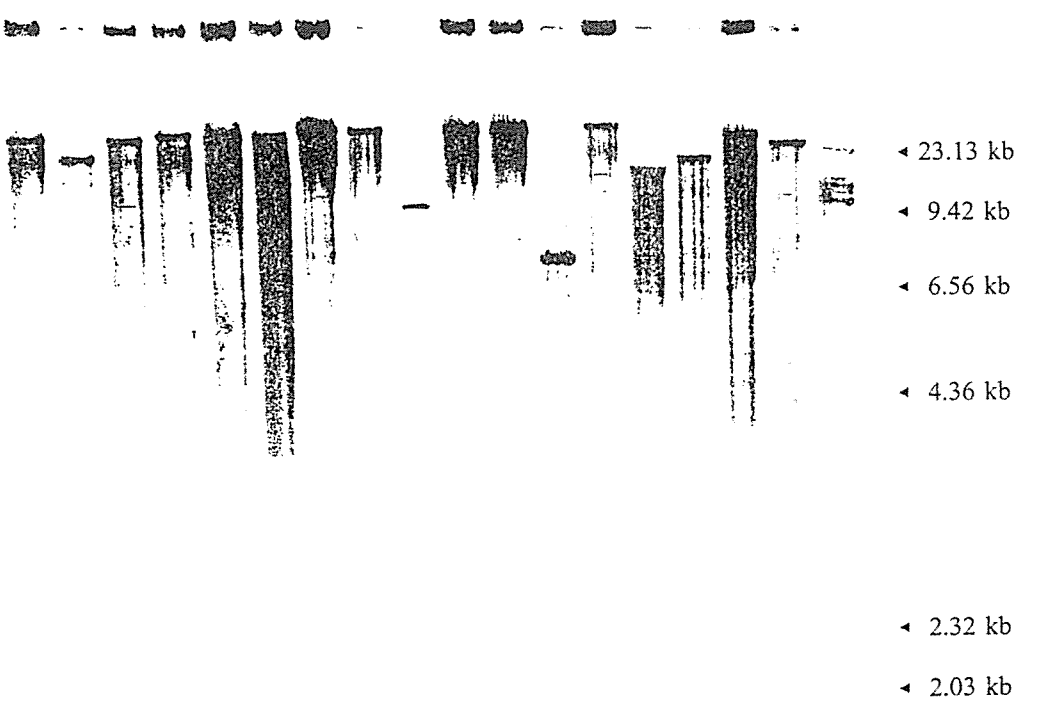


Figure 3.5.2b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20



3.6 Slot Blot Analysis of Exconjugants

There are a number of issues arising from Section 3.5 that require further investigation. The non-random insertion of Tn*5* into pOR168d plasmid needs closer examination, in the hope of finding a plausible mechanism for what appears to be a 'hot spot' for the Tn*5* insertion. Is there any significant sequence homology between pSUP1011 and pOR168d which could serve as a recombination site for the insertion of vector DNA?

Also, it must be remembered that recombination events such as inverse transposition or vector integration, or even RecA-dependent general recombination events, can occur on the introduction of a Tn*5*-bearing vector into a new host; even though such events may occur at frequencies lower than conservative transposition events (Simon *et al.*, 1986; Berg, 1990). The occurrence of such events could help explain the observed alterations to plasmid profiles. Inverse transposition involves cleavage at the IE, rather than OE sequences of Tn*5*, and results in the insertion of vector DNA without the Tn*5* element. Initially, the occurrence of this event was considered of no consequence for the exconjugants screened because only Nm^R-exconjugants were isolated. Later it was considered that in the event of multiple insertion events, insertion of the vector by an inverse transposition would not be apparent.

Total pSUP1011 vector integration into a native OR168 replicon would not be detected simply by screening for the presence of Tn*5*. The only way that events involving either total vector integration or inverse transposition could be detected is to screen for them using pSUP1011 vector-specific DNA sequences other than the Tn*5* as probe. Both recombination events have the possibility of inserting DNA sequences from pSUP1011.

As part of investigating these issues, a more definitive account must be made of the fate of the entire pSUP1011 vector following its conjugative transfer to OR168. This 12.6-kb construct is a composite plasmid consisting of the 4.2-kb pACYC184 vector with a 2.6-kb RP4-derived *oriT*-containing *Sau*3A fragment cloned into the unique *Bam*HI site within the tetracycline resistance gene, resulting in its inactivation. The 5.8-kb transposon Tn5 is contained within the still functional RP4-*oriT* fragment (Simon *et al*, 1983a).

The pACYC184 vector is derived from the narrow host-range enteric replicon p15A (Chang & Cohen, 1978). The pSUP1011 vector carries the origin of replication from p15A. Its independent persistence in the OR168 exconjugants has been rejected on the evidence of the antibiotic resistance results shown in Section 3.4 (namely

chloramphenicol sensitivity). But if recombination events other than conservative transposition of Tn*5* have occurred involving pSUP1011, they will not necessarily be revealed by tests based on the phenotypic expression of one gene. Genotype analysis of the exconjugants for pSUP1011 DNA sequences is needed to investigate the presence of any pSUP1011-derived DNA other than Tn*5*.

The slot-blot technique provides a rapid qualitative screening method for the presence of specific DNA sequences in total genomic DNA preparations. Whenever slot-blots were used in this study the DNA concentration of the samples was first measured to ensure the same amount was applied for each sample. This was to assist in the quantitative analysis of the results, with the intensity of the hybridising signal being proportional to the amount of target DNA present in the sample for each blot. This also avoided the chance of false-negatives, caused by insufficient target DNA being present to give a clear positive signal during probe detection.

Total genomic DNA was prepared using the methods detailed in Section 2.10.1. Four replica slot-blots containing 1 μg of genomic DNA from each exconjugant as well as both donor and recipient parents were then prepared as detailed in Section 2.9. Total genomic DNA from the strains from which the probe DNA originated were included as controls. The blots were then probed for pSUP1011 sequences which may have been incorporated into the exconjugants.

The pSUP1011 DNA sequences probed for included the following:

1. Tn*5*; using the DIG-labelled internal 3.4-kb *Hin*dIII fragment from pKan2.
2. RP4-*mob* fragment; using the DIG-labelled 3.1-kb *Hin*dIII/*Eco*RI fragment from pSUP202 (construct from 6.0-kb pBR325 with a 1.9-kb RP4-derived *mob*-containing fragment).
3. pACYC184; using the DIG-labelled 4.0-kb plasmid isolated from *E. coli* 402A.
4. pSUP1011 in it's entirety; using the DIG-labelled 12.6-kb plasmid isolated from *E. coli* PN302.

Collectively the first three probes cover sequences for almost the entire pSUP1011 vector. The probe DNA preparation and labelling are detailed in Sections 2.11 and 2.12. The probe origins were carefully chosen to avoid ambiguity in the hybridisation results due to sequences from other replicons making up the pSUP1011 being present in the probe. This was the main consideration for choosing the RP4-*mob* fragment from pSUP202. Besides being bound by unique restriction sites which aided the quick preparation of clean DNA, the pSUP202 is a pBR325-based vector and does not contain the transposon Tn*5*.

The possibility of sequence homology between OR168 and the pSUP1011-specific RP4-*mob* fragment has to be investigated in determining if homologous recombination is involved in the observed plasmid alterations. In pSUP1011 the RP4-*mob* fragment is 2.6 kb long, while in pSUP202 the RP4-*mob* fragment is 1.9 kb long. Because the two RP4-*mob* fragments are not identical (one is 700 bp longer), the likelihood of any sequence homology cannot be determined by checking homology to the pSUP202 probe alone. But the RP4-*mob* fragment cannot be easily excised from the pSUP1011 without inclusion of adjoining replicon DNA sequences. In pSUP1011, Tn*5* has been inserted into the RP4-*mob* fragment, so the risk of contaminating a pSUP1011-derived RP4-*mob* probe with Tn*5* sequence is high and this invalidates its use for investigating the question of possible sequence homology between OR168 and pSUP1011. The entire pSUP1011 vector was used as probe DNA to examine any sequence homology between OR168 and the pSUP1011-specific RP4-*mob* sequence, as well as for the pACYC184 and the Tn*5* sequences.

By comparing the hybridisation results of the pSUP1011 probe with the other probes covering specific pSUP1011 sequences (namely, pACYC184, the pKan2 and the 1.9-kb RP4-*mob* fragment) some useful but limited conclusions can be made about sequence homology between the donor and recipient strains. The results of probing with these four plasmid-derived probe DNA sequences are summarised in Table 3.6.1.

The slot-blot hybridisations of total genomic DNA from OR168 showed no sequence homology to any of the probes, including the entire pSUP1011 probe. From this result it can be concluded that there are no significant regions of homologous DNA between the recipient strain OR168 and the donor Tn*5*-carrying plasmid pSUP1011. This eliminates homologous cross-over as an explanation for plasmid 'd' as a target preference and by extension, the involvement of homologous recombination in the observed rearrangements.

As already demonstrated in Section 3.5, the slot-blot results show that each exconjugant contains the transposon Tn*5*. For each exconjugant, hybridisation to the Tn*5* probe has been detected, as was expected.

The slot-blot hybridisation results also revealed no exconjugant had any sequence homology to the pACYC184 probe. This confirms definitively the results observed in Section 3.4, namely the absence of phenotypic expression of chloramphenicol resistance. The pACYC184 probing result also clearly shows no evidence of the total insertion of the pSUP1011 suicide vector into the genome of any exconjugant.

Screening the exconjugants for DNA sequence homology to all the pSUP1011 sequence would reveal any incidence of either vector integration or vector transposition. The slot-blot hybridisation results show no evidence for either event, which discounts them as the cause for any of the observed plasmid profile alterations.

However, the slot-blot hybridisation results do show sequence homology between DNA from *E. coli* PN338 and DNA from *E. coli* 402A. The DIG-labelled pACYC184 probe hybridised to total genomic DNA from *E. coli* PN338/pSUP202. This happened because both pSUP202 and pACYC184 vector constructs contain a Tc^R gene derived from pSC101. The DIG-labelled *Hin*dIII/*Eco*RI pSUP202 probe hybridised to total genomic DNA from *E. coli* 402A/pACYC184, again because both contain some DNA sequence from pSC101.

The most interesting result revealed by the slot-blot analysis is the first hard evidence of events other than conservative transposition having occurred with the introduction of Tn*5* into the OR168 genome. A total of 33 of the 53 exconjugants screened contain DNA sequences homologous to the RP4 specific *mob*-site. Of the 20 that do not, all but two carry the Tn*5* element on their chromosome. The two exceptions (one from each cross) are DH120 and DH225. Both have a plasmid-borne Tn*5* element.

As discussed in Section 3.2.1, with DH120 the plasmid bearing the Tn*5* element, pDH120d, shows very little change in mobility compared to all other 'd' plasmids containing Tn*5*. For DH120 the change in mobility equates to an increase in plasmid size of about 6 kb. The lack of any other DNA sequence homology to the pSUP1011 vector indicates that the change in mobility in pDH120d is due solely to the acquisition of Tn*5* by transposition.

For DH225, no change in mobility of the plasmid containing the Tn*5* element is detected. This is because a 5.8 kb increase in size for a 570 kb plasmid is below the sensitivity of the gel method used for sizing the plasmids. But by extension, the reasoning used to call the acquisition of Tn*5* in DH120 'transposition' can be used for DH225. In both cases there is a lack of DNA sequence homology to pSUP1011, except for the transposon Tn*5*.

The slot-blot results for the 20 exconjugants hybridising only to probes containing Tn*5* sequence suggests that they have acquired Tn*5* as the result of a true transposition event. This conclusion still doesn't explain the resultant plasmid rearrangements seen in the plasmids not bearing Tn*5*, in particular the presumed plasmid derivatives of pOR168b.

Assuming the chromosome size of OR168 is of the same order of magnitude as *R. meliloti*, which has been estimated to be about 3.5 Mb (Honeycutt *et al.*,1993), and the plasmid complement of OR168 has been calculated to make up approximately 1 Mb in Section 3.3, then approximately 22% of random Tn*5* transposition events should involve plasmid DNA as the target. In the two crosses analysed the frequency of true transposition events involving plasmid DNA is only 10% (2 plasmid targets of 20 normal transposition events). This lower than expected incidence may simply be the consequence of small sample size, meaning no conclusion with any confidence can be drawn from the events observed.

The presence of RP4-*mob* fragments show clearly that events other than conservative transposition of Tn*5* have occurred in the recipient OR168. But the acquisition of the RP4-*mob* fragments does not account for the estimated change in size of pOR168d from its original 22 kb to 40 kb. The Tn*5* is 5.8 kb long, and the RP4-*mob* fragment in pSUP1011 is only 2.6 kb. So a further 9.6 kb of DNA in the 40-kb plasmid cannot been accounted for, assuming the integration of the entire RP4-*mob* DNA fragment.

At this stage the physical location of the RP4-*mob* fragment in exconjugants containing it has not even been determined. It is expected that the plasmids bearing the Tn*5* also contain the RP4-*mob* fragment, since the Tn*5* is located within the RP4-*mob* fragment on pSUP1011. If this is the case, some mechanism other than conservative transposition may be involved in the integration of a portion of the vector DNA sequence seen in the many anomalous OR168 exconjugants.

In summary, the conclusions that can be drawn from the results displayed from probing the Eckhardt gels and from the slot-blots of exconjugant genomic DNA are as follows:

i. There is a total lack of sequence homology between the DNAs of OR168 and pSUP1011. This eliminates the involvement of homologous recombination in the Tn*5* insertion 'hot spot' observed for the plasmid pOR168d.
ii. Two different products from insertion events are seen. These are the insertion of Tn*5* alone, and alternatively, the combined insertions of Tn*5* and RP4-*mob* fragments within the same genome.
iii. There is a strong correlation between the size of the Tn*5*-containing *Eco*RI fragment and the involvement of plasmid pOR168d as the replicon target for Tn*5* insertion. The insertions of RP4-*mob*::Tn*5* fragments coincides with the Tn*5* being situated in large (>23.1 kb) *Eco*RI fragments of plasmid 'd'. Further restriction digests are needed to investigate and characterise both types of insertion product.

KEY to TABLE 3.6.1: The table opposite lists the presence or absence of specific replicon sequences, that make up pSUP1011, in each of the isolated exconjugants, as well as in both the recipient parent, OR168, and the donor parent, *E. coli* PN302. The other *E. coli* strains that were the source of probe DNA were included as hybridisation controls, since the presence or absence of the probed sequences is already known in these. Each column is labelled with the particular sequence that was looked for in the isolates. For identifying the presence of Tn*5*, the internal 3.5-kb *Hin*dIII fragment of Tn*5* from pKan2 was used as probe DNA. For identifying the presence of any pACYC184 sequences, the entire pACYC184 plasmid from *E. coli* 402A was used as probe DNA. For identifying the presence of the RP4-derived DNA from pSUP1011, the RP4-*mob* fragment from pSUP202 was used as probe DNA. Note, the RP4-derived DNA fragment is a 1.9 kb long in pSUP202, whilst in pSUP1011 the RP4-derived DNA fragment is a 2.6 kb long. The entire pSUP1011 plasmid from *E. coli* PN302 was also used as a probe, both as a control and to examine any sequence homology between OR168 and pSUP1011.

Symbols: +, presence of the sequence indicated; -, absence of the sequence indicated;
*, the Class number is an arbitrarily conferred number that groups exconjugants displaying the same plasmid profile to the same class. Exconjugants of Class I form the most numerous class, consisting of a total of 23 exconjugants from both crosses. The Class I exconjugants all contain five plasmids, which are ~1000 kb, 570 kb, 295 kb, 163 kb, and 40 kb in size. The Class II make up the next most predominant class, consisting of 14 exconjugants from both crosses. Again, the Class II exconjugants contain five plasmids, but are ~1000 kb, 570 kb, 295 kb, 163 kb, and 22 kb in size. The Class III exconjugants, by default, include all those which do not fit into the two previous classes. The class is made up of 16 exconjugants, each with a unique plasmid profile.
†, the Tn*5* site for each isolate was determined from the probed Eckhardt Southerns shown in Figures 3.3.1 to 3.3.9.

Table 3.6.1: Summary of hybridisation results of Eckhardt gels and slot-blots

Isolate	Class*	Tn*5* Site†	Tn*5*	RP4-*mob*	pACYC184	pSUP1011
Exconjugants:						
DH101	I	plasmid d	+	+	-	+
DH102	I	plasmid d	+	+	-	+
DH103	I	plasmid d	+	+	-	+
DH104	I	plasmid d	+	+	-	+
DH105	I	plasmid d	+	+	-	+
DH106	III	plasmid d	+	+	-	+
DH107	I	plasmid d	+	+	-	+
DH108	III	plasmid d	+	+	-	+
DH109	II	megaplasmid	+	-	-	+
DH110	II	chromosome	+	-	-	+
DH111	I	plasmid d	+	+	-	+
DH112	III	chromosome	+	-	-	+
DH113	I	plasmid d	+	+	-	+
DH114	I	plasmid d	+	+	-	+
DH115	II	chromosome	+	-	-	+
DH116	III	plasmid d	+	+	-	+
DH117	II	chromosome	+	-	-	+
DH118	II	chromosome	+	-	-	+
DH119	I	plasmid d	+	+	-	+
DH120	III	plasmid d	+	-	-	+
DH121	I	plasmid d	+	+	-	+
DH122	III	plasmid c	+	+	-	+
DH123	II	megaplasmid	+	-	-	+
DH124	III	plasmid d	+	+	-	+
DH125	III	plasmid d	+	+	-	+
DH126	II	plasmid c	+	-	-	+
DH127	I	plasmid d	+	+	-	+
DH201	I	plasmid d	+	+	-	+
DH202	II	chromosome	+	-	-	+
DH203	II	chromosome	+	-	-	+
DH204	III	chromosome	+	-	-	+
DH205	III	plasmid d	+	+	-	+
DH206	I	plasmid d	+	+	-	+
DH207	II	chromosome	+	-	-	+
DH208	II	chromosome	+	-	-	+
DH209	III	chromosome	+	-	-	+
DH210	II	chromosome	+	-	-	+
DH211	III	chromosome	+	-	-	+
DH212	III	plasmid d	+	+	-	+
DH213	I	plasmid d	+	+	-	+
DH214	III	chromosome	+	-	-	+
DH215	I	plasmid d	+	+	-	+
DH216	III	plasmid d	+	+	-	+
DH217	I	plasmid d	+	+	-	+
DH218	I	plasmid d	+	+	-	+
DH219	I	plasmid d	+	+	-	+
DH220	I	plasmid d	+	+	-	+
DH221	II	chromosome	+	-	-	+
DH222	III	chromosome	+	-	-	+
DH223	I	plasmid d	+	+	-	+
DH224	I	plasmid d	+	+	-	+
DH225	II	plasmid a	+	-	-	+
DH226	I	plasmid d	+	+	-	+
Parental Strains:						
OR168			-	-	-	-
PN302			+	+	+	+
Controls:						
PN338			-	+	+	+
402A			-	+	+	+

3.7 Further Investigation of Tn*5* Insertion Site and Related Changes in Plasmid Mobility

The initial assumption made, prior to probing Eckhardt gels and genomic restriction fragments for the transposon Tn*5*, was that the observed alterations in plasmid mobility were probably due to the acquisition of Tn*5* by those plasmids. The information obtained from probing both the Eckhardt gels and total genomic restriction fragments for Tn*5* (discussed in Section 3.5) showed only a single copy of the transposon within each exconjugant genome. The results also revealed that the mobility of some plasmids has been altered without actually carrying Tn*5*. This ruled out the presence of Tn*5* in a particular plasmid as being solely responsible for alterations in the mobility of that plasmid. Further more, the plasmid sizing exercise (see Tables 3.3.1 to 3.3.9) has revealed that those plasmids now carrying Tn*5* have usually increased in size by much more than 5.8 kb (the size of Tn*5*). These observations cannot be explained as the result of conservative transposition alone.

In an attempt to explain the alterations to the plasmid profiles from the hybridisation information, two possible events that could be causing alterations were investigated. One of these events may involve a recombination activity other than transposition and is suggested by the slot blot hybridisation results which show the insertion of pSUP1011-derived sequences in addition to the transposon. This observation is investigated and discussed in detail in Section 3.8.

The other type of event that may explain some of the alterations to plasmid profiles is that they are the result of topological changes in the plasmid DNA after their acquisition of Tn*5*. Resultant changes in plasmid topology may alter susceptibility to cleavage by some restriction endonucleases. This may be the basis for the unusually large *Eco*RI restriction fragments observed with the Tn*5* probing of the genomic restriction fragments from exconjugants with plasmid-borne Tn*5* elements. The possibility of this being the cause of alterations to plasmid mobility is discussed in detail below, in Section 3.7.1.

Whatever the actual cause for the alterations in plasmid mobility, the 22-kb pOR168d appears to be a preferred target for transposon insertion. This generally results in a relatively large increase in size for the plasmid. The set of experiments undertaken in this section and Section 3.8 were aimed at determining which of the two suggested activities have resulted in, or at least contributed to, the observed plasmid modifications.

A total of 27 exconjugants (15 from Cross 1, 12 from Cross 2) were chosen for further restriction pattern analysis of their Tn*5* insertion sites. The exconjugants used are listed in Tables 3.3.7 to 3.3.9. They include examples of each of the plasmid profile patterns depicted in Figures 3.3.10 and 3.5.1. This meant including all the exconjugants belonging to Class III. More than one example of the two predominant profile classes (Classes I and II) were also included from each cross. This ensured screening of all variables observed from the two crosses. It also meant that exconjugants displaying the same plasmid profiles could be checked to see if in fact they could be considered clonal derivatives. Collectively, it allowed any common or discriminating patterns to be recognised, regarding the insertion of Tn*5*, especially into the 22-kb pOR168d plasmid. The broader scope of the later restriction pattern analysis, due to the wider number of exconjugants analysed and the wider number of restriction enzymes used, will help clarify the ambiguous results from the initial restriction pattern analysis discussed in Section 3.5.

3.7.1 Detailed Analyses of Exconjugant Total Genomic DNA Restriction Patterns

Examination of the genomic DNA restriction results in Section 3.5.2.1 has revealed that the *Eco*RI fragment hybridising to the Tn*5* probe is universally in excess of 23 kb when the site of Tn*5* insertion is plasmidic, irrespective of the plasmid target. This contrasts strongly to the pattern of *Eco*RI fragment sizes seen when the target for Tn*5* insertion is the chromosome. The restriction enzyme *Eco*RI has an A+T-rich 6-base recognition sequence. It is expected to cleave DNA with a 50% G+C content every 4^6 (~4.1 kb) bases on average. Analysis of *Rhizobiaceae* genomic sequences shows that they are G+C-rich bacteria. This means cleavage by a restriction enzyme with an A+T-rich target site is expected to arise less frequently. If this is the case it may be the simple explanation for the observed genomic DNA restriction results when using *Eco*RI. To determine whether the unusually large fragment size was due to unconventional topological modifications in the DNA after the insertion of Tn*5* which prevented binding of the restriction enzyme, other restriction enzymes with recognition sites within Tn*5* were chosen. If DNA structure prevented binding of the restriction enzyme the predicted number of hybridising bands will not be seen.

The restriction enzyme *Bam*HI has a single G+C-rich 6-base recognition site within Tn*5* giving two fragments which hybridise to the pKan2-derived Tn*5* probe. The restriction enzyme *Hin*dIII has an A+T-rich 6-base recognition sequence at two sites within Tn*5* giving a single internal fragment which will hybridise to the pKan2-derived Tn*5* probe.

Using methods detailed in Section 2.10, genomic DNA was extracted from the 27 exconjugants and OR168 listed in Tables 3.3.7 to 3.3.9 and digested. The three restriction enzymes *Bam*HI, *Eco*RI, and *Hin*dIII were individually used to cleave aliquots of the purified genomic DNA. Then 1 μg of the digested genomic DNA was separated by gel electrophoresis and Southern blots prepared from the gels.

The Southern blots were probed for Tn*5* using the internal 3.5-kb *Hin*dIII fragment of pKan2. The Figures 3.7.1a to 3.7.9a show the gel results, with the Tn*5* probing results for the nine gels shown in Figures 3.7.1b to 3.7.9b, respectively. Tables 3.7.1 to 3.7.3 also list the size of the DNA fragments hybridising to the Tn*5* probe for each exconjugant screened.

All enzymes gave the expected number of fragments hybridising to Tn*5*. On all blots the *Hin*dIII restriction fragments for all exconjugants showed a single 3.4-kb band hybridising to the Tn*5*-probe irrespective of the plasmid profile or the target of Tn*5* insertion. The *Bam*HI restriction digests for the exconjugants gave the two bands expected. This result shows that the restriction enzymes are able to cleave with equal ease within the site of Tn*5* insertion regardless of the target. This observation allows the rejection of the hypothesis that alterations in plasmid mobility are due to conformational changes in the plasmid molecules which prevent recognition and cleavage by restriction enzymes.

Analysis of the sizes of the DNA fragments hybridising to the Tn*5* probe have revealed some interesting results. The exconjugants that contain Tn*5* on plasmid 'd' all appear to generate the same fragment sizes for each of the three restriction enzymes used. This indicates the same pattern of restriction sites for these three enzymes over an estimated 27-kb length of DNA within plasmid 'd' containing the Tn*5*. This suggests the same insertion site for Tn*5* on plasmid 'd' for each exconjugant regardless of their cross origin, with the exception of DH120, which, unlike the other exconjugants carrying Tn*5* in plasmid 'd', does not contain the RP4-*mob* fragment. The simplest interpretation of this variant pattern is that it results from a different site of insertion, presumably the consequence of a conventional conservative transposition.

Another observation worth noting is the sizes of the restriction fragments for DH122 and DH126. Both of these exconjugants carry Tn*5* in plasmid 'c', although the sizes of their restriction fragments are distinctly different. This suggests different insertion sites for the Tn*5* in plasmid 'c' for these two exconjugants. But the fragment sizes for DH122, which contains the RP4-*mob* fragment, are the same as for exconjugants carrying Tn*5* on plasmid 'd', and also containing the RP4-*mob* fragment. Does this suggest similar sites of insertion for Tn*5* on plasmids 'c' and 'd' in these cases? If this

is so, it further suggests the presence of the same DNA sequences repeated on different replicons within OR168. This may provide an explanation for the observed plasmid instability in the exconjugants. Repeated DNA sequences on different replicons may provide sites for homologous recombination to occur.

Figure 3.7.1: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.1a. Agarose gel electrophoretic separation of *Eco*RI-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.1b. Southern hybridisation of the gel shown above, in Fig. 3.7.1a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH101	plasmid 'd'	+
	2	DH106	plasmid 'd'	+
	3	DH108	plasmid 'd'	+
	4	DH109	megaplasmid	-
	5	DH112	chromosome	-
	6	DH113	plasmid 'd'	+
	7	DH115	chromosome	-
	8	DH116	plasmid 'd'	+
	9	DH118	chromosome	-
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.1a: Gel electrophoresis of total genomic DNA digested with *Eco*R1

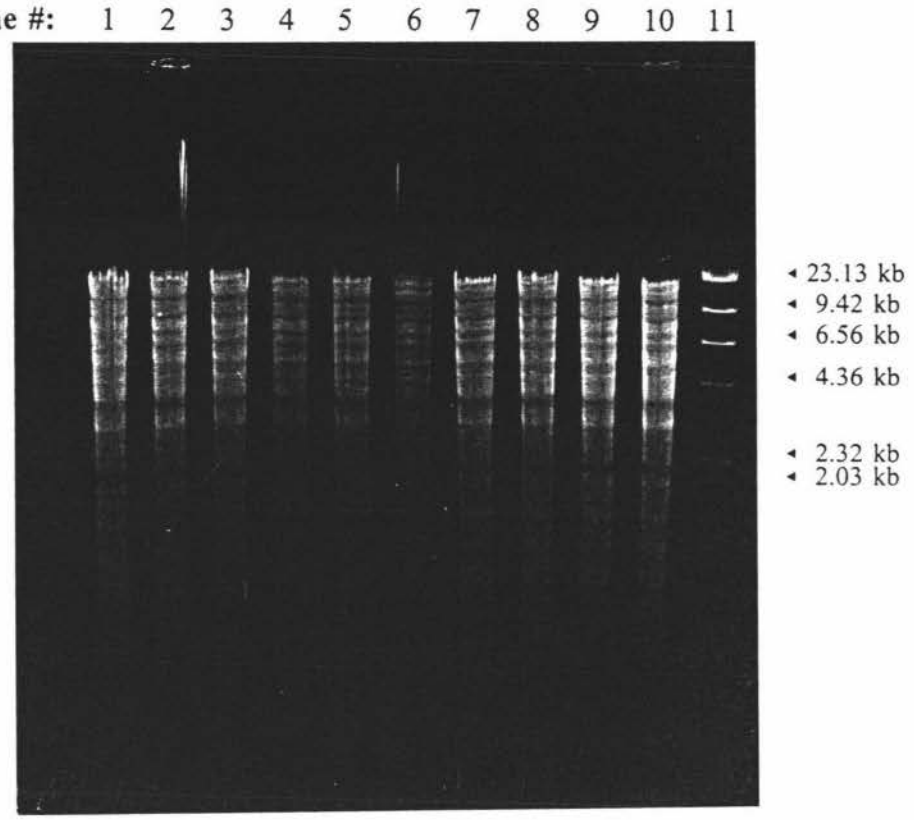


Figure 3.7.1b: Southern blot of above gel probed for Tn*5*

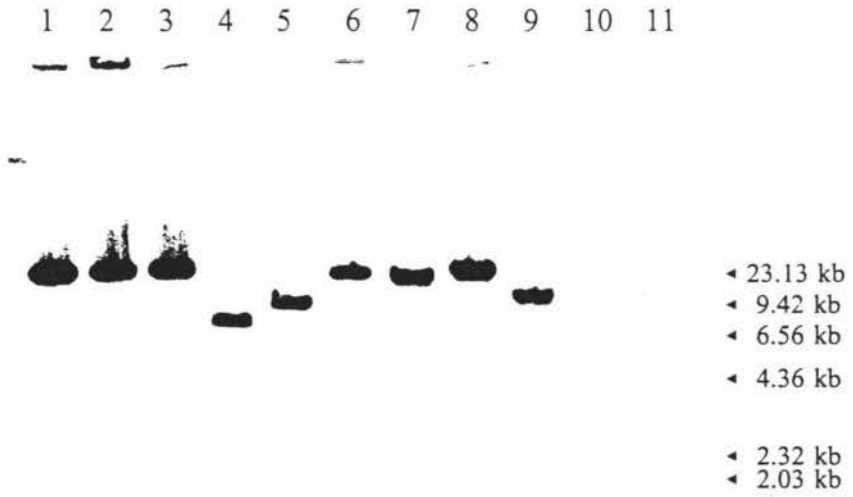


Figure 3.7.2: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.2a. Agarose gel electrophoretic separation of *Bam*HI-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.2b. Southern hybridisation of the gel shown above, in Fig. 3.7.2a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH101	plasmid 'd'	+
	2	DH106	plasmid 'd'	+
	3	DH108	plasmid 'd'	+
	4	DH109	megaplasmid	-
	5	DH112	chromosome	-
	6	DH113	plasmid 'd'	+
	7	DH115	chromosome	-
	8	DH116	plasmid 'd'	+
	9	DH118	chromosome	-
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.2a: Gel electrophoresis of total genomic DNA digested with *Bam*HI

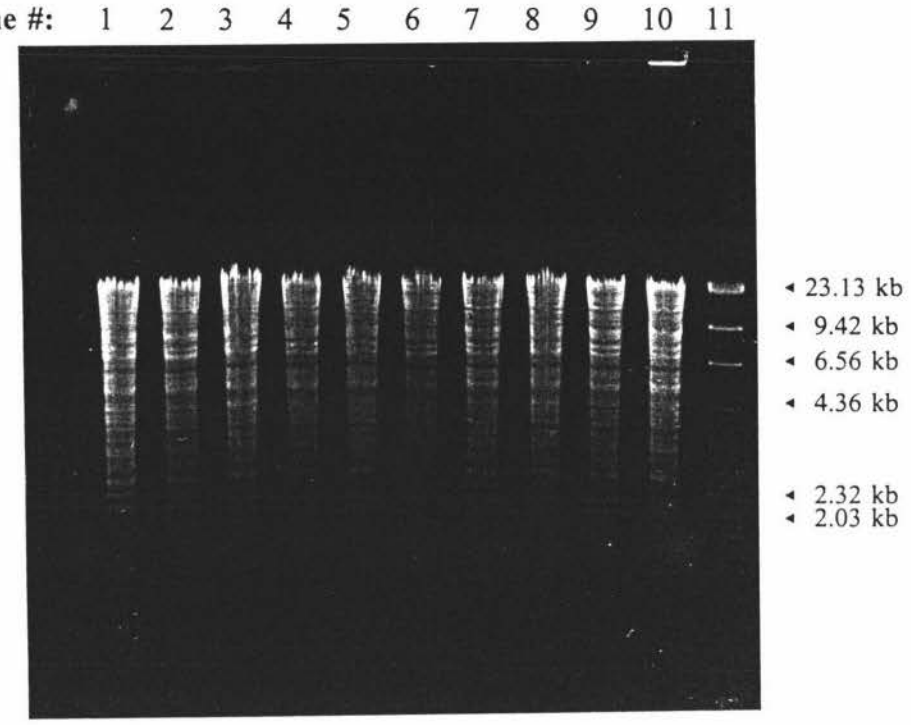


Figure 3.7.2b: Southern blot of above gel probed for Tn*5*

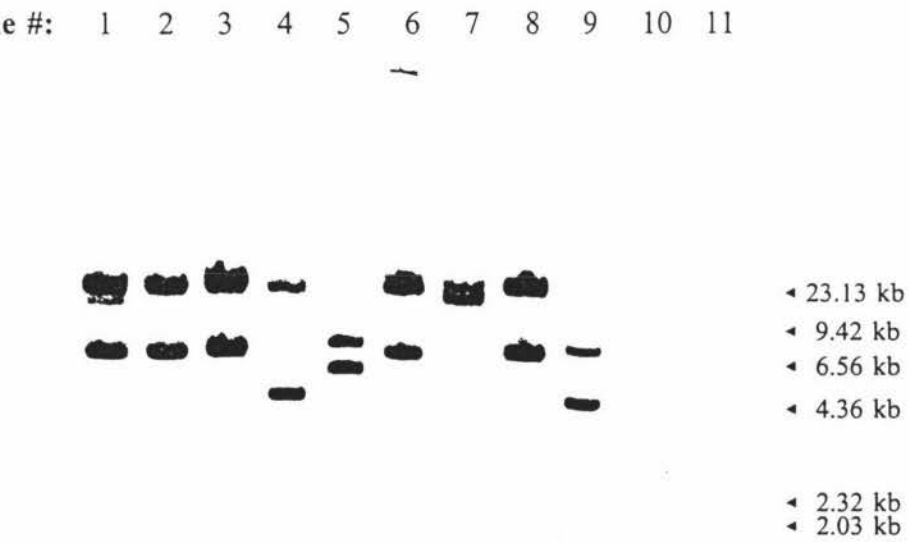


Figure 3.7.3: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.3a. Agarose gel electrophoretic separation of *Hin*dIII-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.3b. Southern hybridisation of the gel shown above, in Fig. 3.7.3a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH101	plasmid 'd'	+
	2	DH106	plasmid 'd'	+
	3	DH108	plasmid 'd'	+
	4	DH109	megaplasmid	-
	5	DH112	chromosome	-
	6	DH113	plasmid 'd'	+
	7	DH115	chromosome	-
	8	DH116	plasmid 'd'	+
	9	DH118	chromosome	-
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Note: The DNA sample of OR168 shown in lane 10 is undigested due to no restriction enzyme in the reaction mix. This has not invalidated the experiment because DNA from the parental strain was only used as a source of control DNA.

Figure 3.7.3a: Gel electrophoresis of total genomic DNA digested with *Hin*dIII

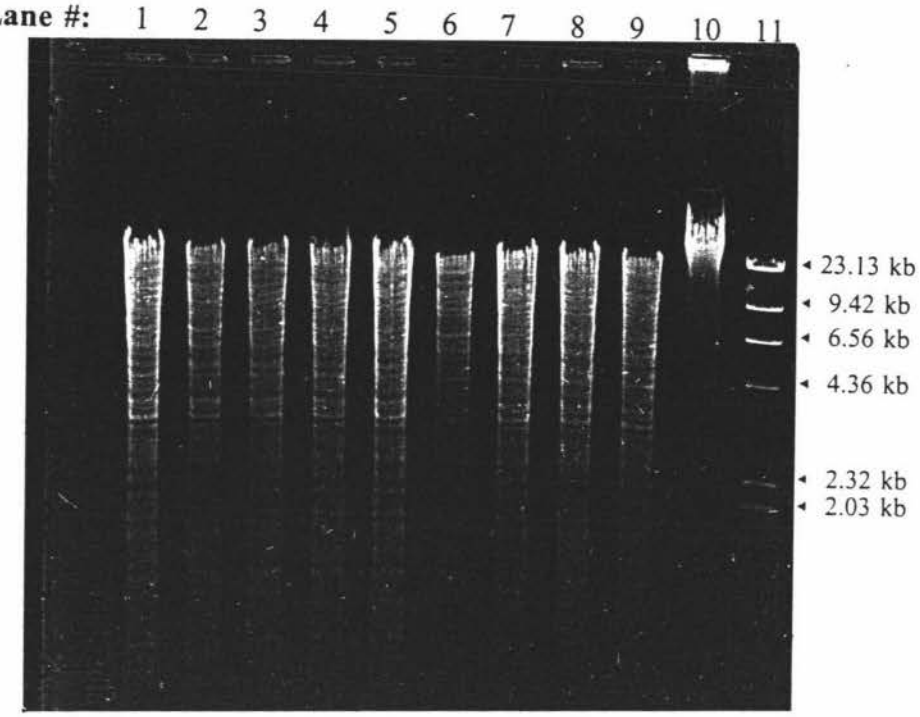


Figure 3.7.3b: Southern blot of above gel probed for Tn*5*

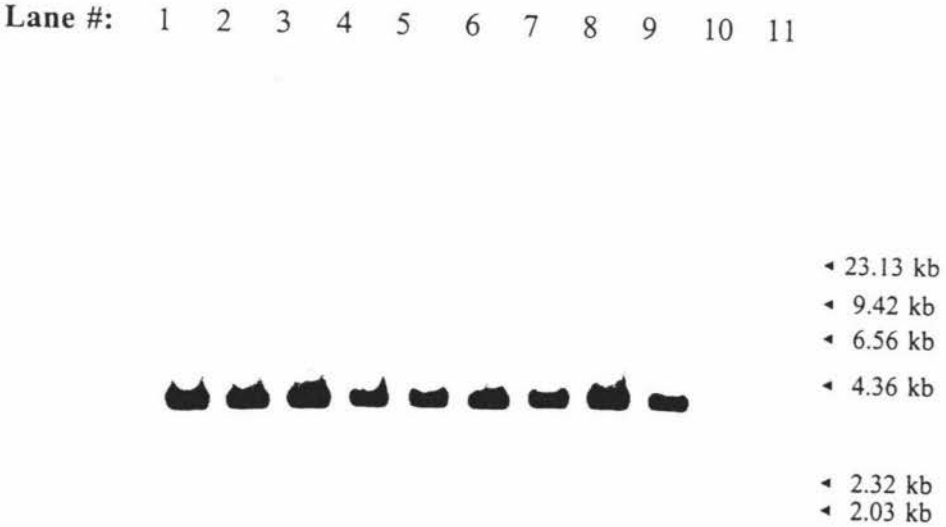


Figure 3.7.4: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.4a. Agarose gel electrophoretic separation of *Eco*RI-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.4b. Southern hybridisation of the gel shown above, in Fig. 3.7.4a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH119	plasmid 'd'	+
	2	DH120	plasmid 'd'	-
	3	DH122	plasmid 'c'	+
	4	DH124	plasmid 'd'	+
	5	DH125	plasmid 'd'	+
	6	DH126	plasmid 'c'	-
	7	DH201	plasmid 'd'	+
	8	DH204	chromosome	-
	9	DH205	plasmid 'd'	+
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.4a: Gel electrophoresis of total genomic DNA digested with *Eco*RI

Lane #: 1 2 3 4 5 6 7 8 9 10 11

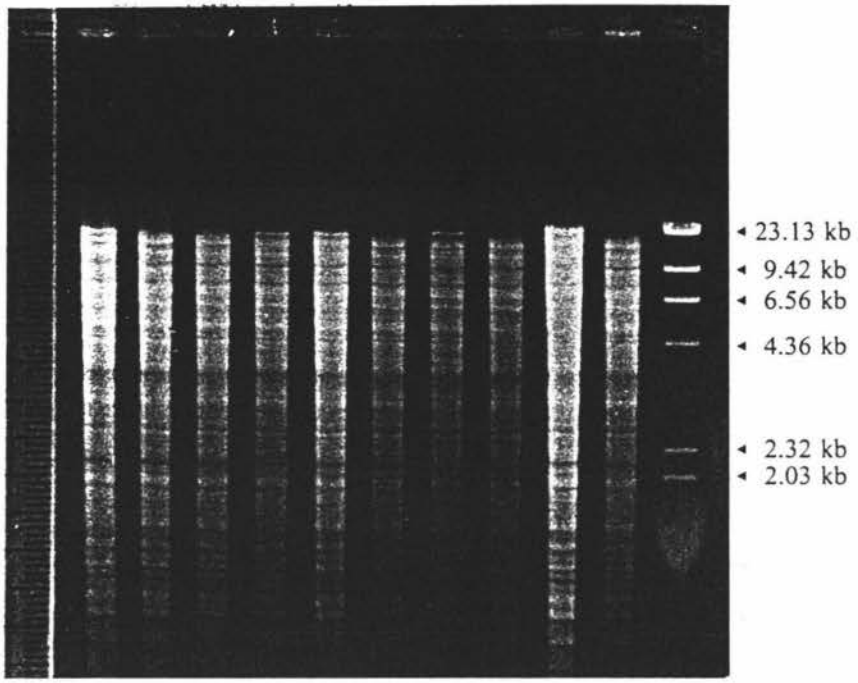


Figure 3.7.4b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10 11

Figure 3.7.5: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.5a. Agarose gel electrophoretic separation of *Bam*HI-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.5b. Southern hybridisation of the gel shown above, in Fig. 3.7.5a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH119	plasmid 'd'	+
	2	DH120	plasmid 'd'	-
	3	DH122	plasmid 'c'	+
	4	DH124	plasmid 'd'	+
	5	DH125	plasmid 'd'	+
	6	DH126	plasmid 'c'	-
	7	DH201	plasmid 'd'	+
	8	DH204	chromosome	-
	9	DH205	plasmid 'd'	+
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.5a: Gel electrophoresis of total genomic DNA digested with *Bam*HI

Lane #: 1 2 3 4 5 6 7 8 9 10 11

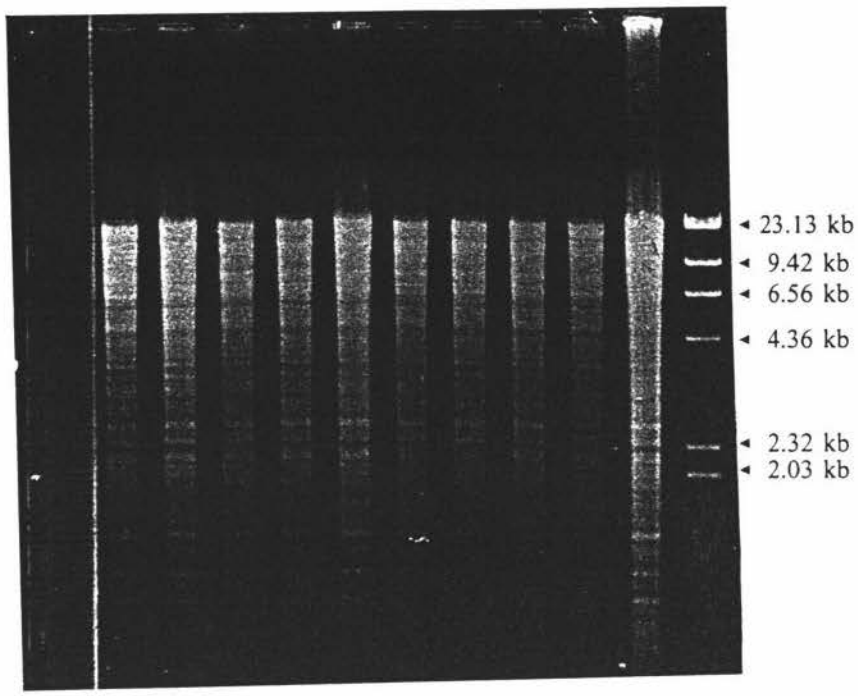


Figure 3.7.5b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10 11

Figure 3.7.6: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.6a. Agarose gel electrophoretic separation of *Hin*dIII-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.6b. Southern hybridisation of the gel shown above, in Fig. 3.7.6a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH119	plasmid 'd'	+
	2	DH120	plasmid 'd'	-
	3	DH122	plasmid 'c'	+
	4	DH124	plasmid 'd'	+
	5	DH125	plasmid 'd'	+
	6	DH126	plasmid 'c'	-
	7	DH201	plasmid 'd'	+
	8	DH204	chromosome	-
	9	DH205	plasmid 'd'	+
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.6a: Gel electrophoresis of total genomic DNA digested with *Hin*dIII

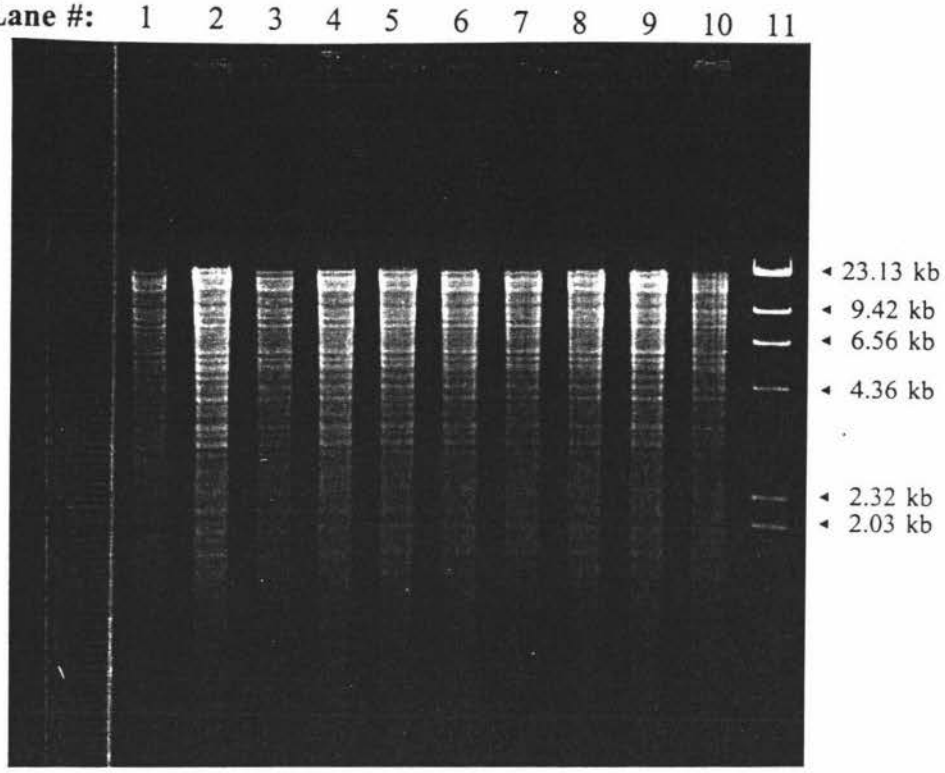


Figure 3.7.6b: Southern blot of above gel probed for Tn*5*

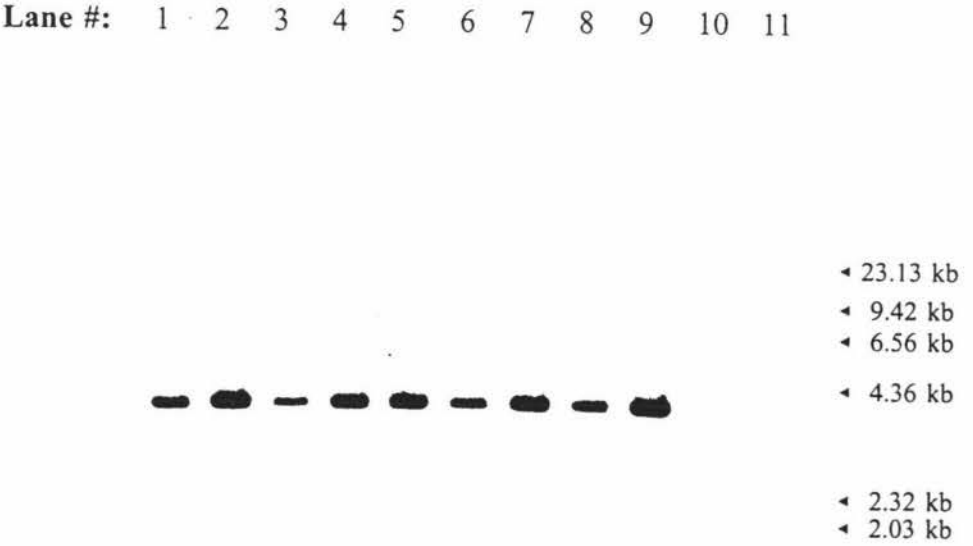


Figure 3.7.7: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.7a. Agarose gel electrophoretic separation of *Eco*RI-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.7b. Southern hybridisation of the gel shown above, in Fig. 3.7.7a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH209	chromosome	-
	2	DH211	chromosome	-
	3	DH212	plasmid 'd'	+
	4	DH213	plasmid 'd'	+
	5	DH214	chromosome	-
	6	DH216	plasmid 'd'	+
	7	DH221	chromosome	-
	8	DH222	chromosome	-
	9	DH225	plasmid 'a'	-
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.7: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.7a. Agarose gel electrophoretic separation of *Eco*RI-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.7b. Southern hybridisation of the gel shown above, in Fig. 3.7.7a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH209	chromosome	-
	2	DH211	chromosome	-
	3	DH212	plasmid 'd'	+
	4	DH213	plasmid 'd'	+
	5	DH214	chromosome	-
	6	DH216	plasmid 'd'	+
	7	DH221	chromosome	-
	8	DH222	chromosome	-
	9	DH225	plasmid 'a'	-
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.7a: Gel electrophoresis of total genomic DNA digested with *Eco*RI

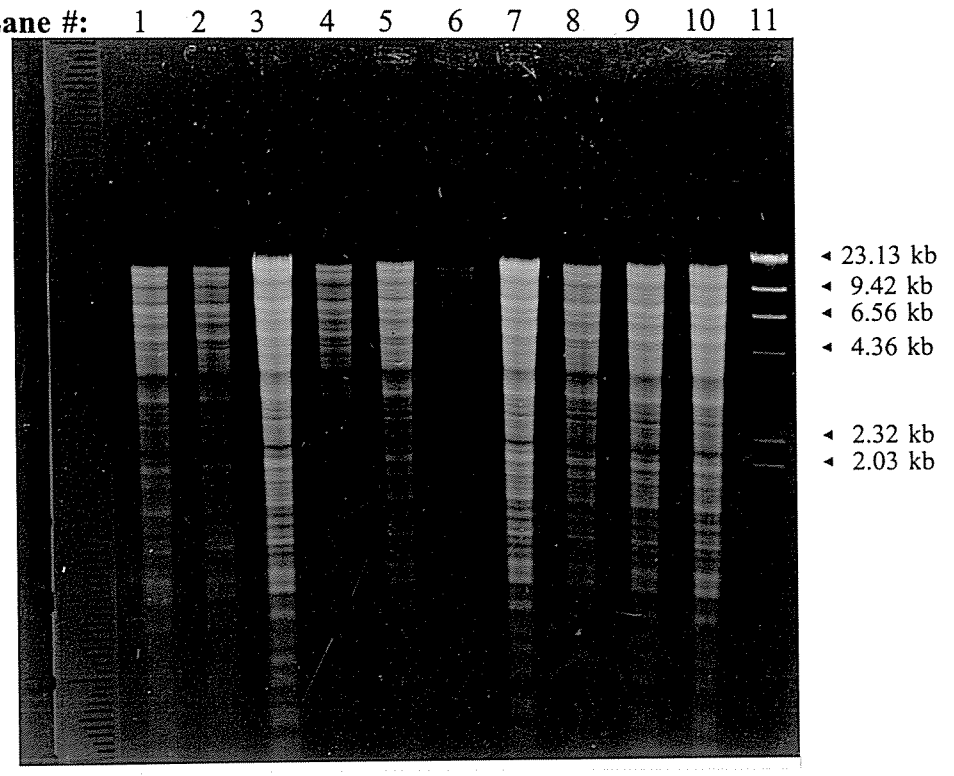


Figure 3.7.7b: Southern blot of above gel probed for Tn*5*

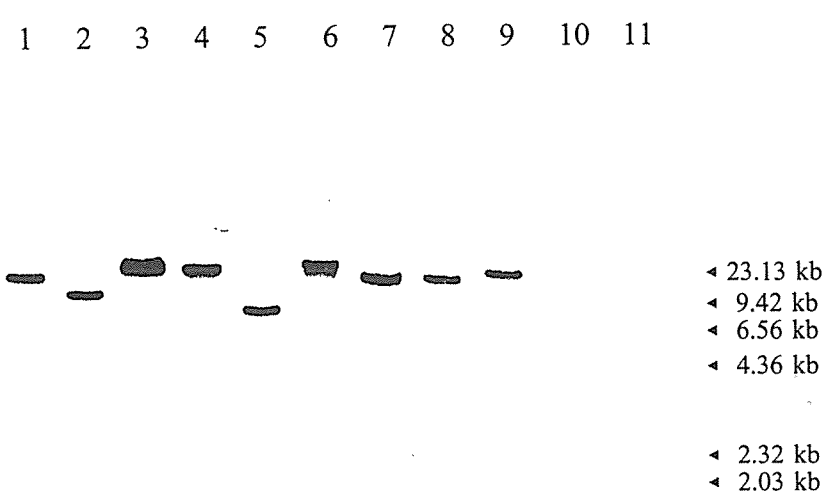


Figure 3.7.8: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.8a. Agarose gel electrophoretic separation of *Bam*HI-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.8b. Southern hybridisation of the gel shown above, in Fig. 3.7.8a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH209	chromosome	-
	2	DH211	chromosome	-
	3	DH212	plasmid 'd'	+
	4	DH213	plasmid 'd'	+
	5	DH214	chromosome	-
	6	DH216	plasmid 'd'	+
	7	DH221	chromosome	-
	8	DH222	chromosome	-
	9	DH225	plasmid 'a'	-
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.8a: Gel electrophoresis of total genomic DNA digested with *Bam*HI

Lane #: 1 2 3 4 5 6 7 8 9 10 11

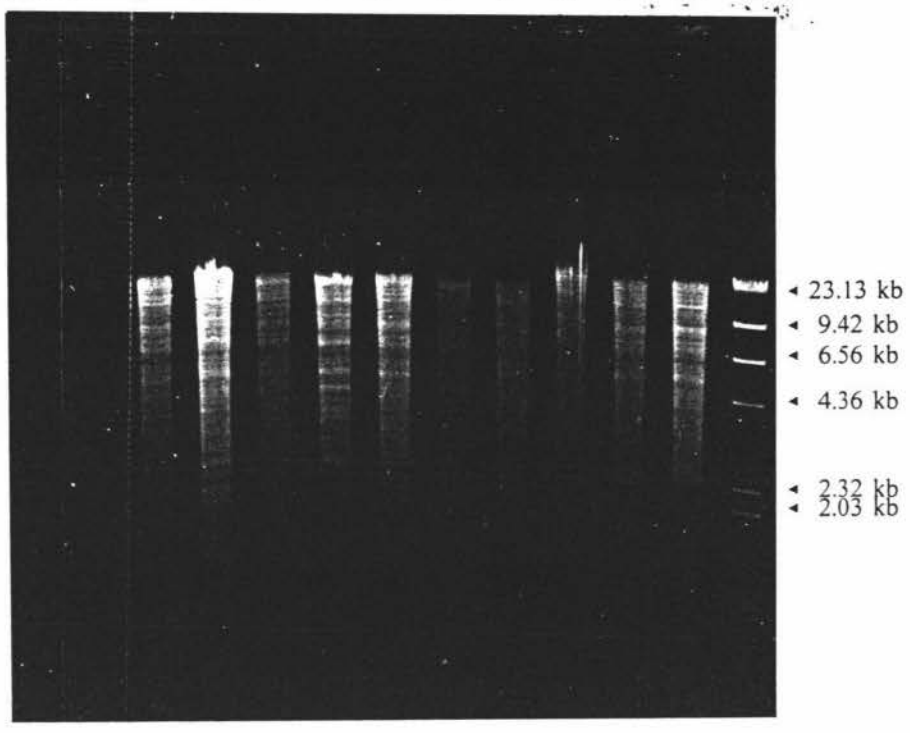


Figure 3.7.8b: Southern blot of above gel probed for Tn*5*

Lane #: 1 2 3 4 5 6 7 8 9 10 11

Figure 3.7.9: Electrophoresis and detection of Tn*5* in digested total genomic DNA from OR168::Tn*5* exconjugants and the OR168 parent.

Figure 3.7.9a. Agarose gel electrophoretic separation of *Hin*dIII-digested genomic DNA from selected OR168::Tn*5* exconjugants and the parental strain, OR168. The gel was stained with ethidium bromide. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

Figure 3.7.9b. Southern hybridisation of the gel shown above, in Fig. 3.7.9a, probed with the internal *Hin*dIII fragment from pKan2 to detect the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section.

		Isolate	Tn*5* Site	RP4-*mob* Fragment
Lane #	1	DH209	chromosome	-
	2	DH211	chromosome	-
	3	DH212	plasmid 'd'	+
	4	DH213	plasmid 'd'	+
	5	DH214	chromosome	-
	6	DH216	plasmid 'd'	+
	7	DH221	chromosome	-
	8	DH222	chromosome	-
	9	DH225	plasmid 'a'	-
	10	0R168	-	-
	11	*Hin*dIII λ DNA size markers		

Figure 3.7.9a: Gel electrophoresis of total genomic DNA digested with *Hin*dIII

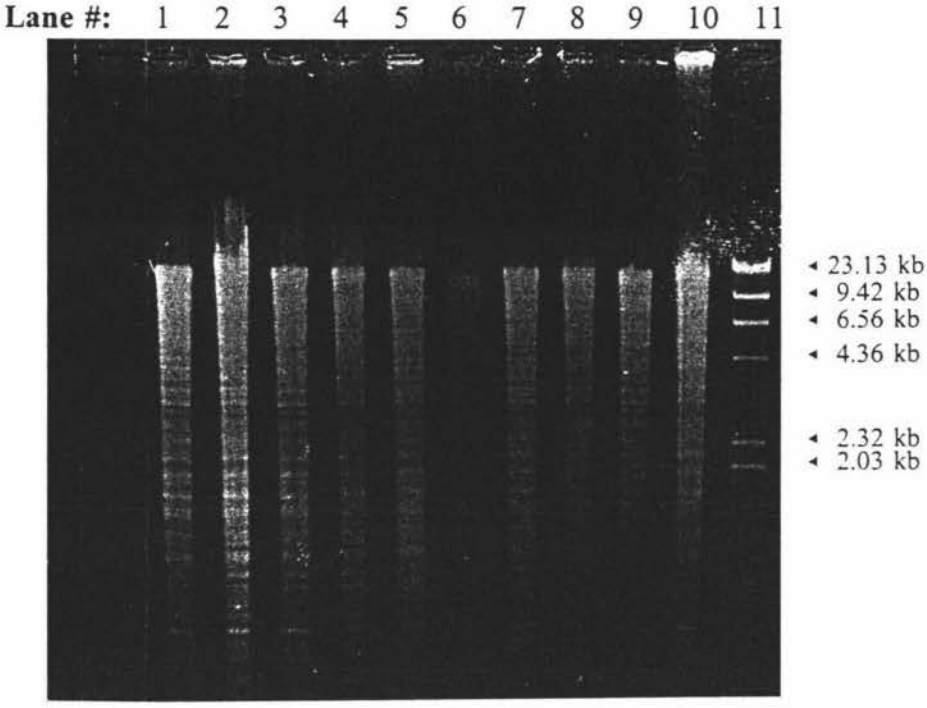


Figure 3.7.9b: Southern blot of above gel probed for Tn*5*

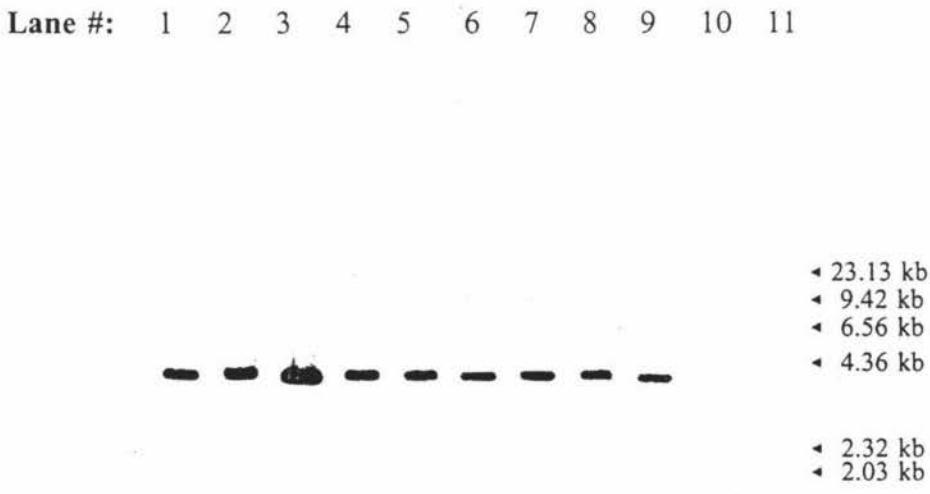


Table 3.7.1: Summary of sizes of restriction fragments hybridising to Tn*5*-probe on Southern blots shown in Figures 3.7.1 to 3.7.3

	EXCONJUGANT:	DH101		DH106		DH108		DH109		DH112		DH113		DH115		DH116		DH118	
Figure	Enzyme	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb
3.7.1b	*Eco*R1	28.0	23.1	28.0	23.1	28.0	23.1	34.7	7.4	32.5	9.0	28.0	23.1	29.0	16.2	28.0	23.1	33.0	9.1
3.7.2b	*Bam*H1	30.5	20.3	30.5	20.3	30.5	20.3	30.0	23.1	37.2	7.6	30.5	20.3	30.5	20.3	30.5	20.3	38.0	7.2
		39.0	6.6	39.0	6.6	39.0	6.6	44.0	4.7	40.8	5.8	39.0	6.6	31.2	19.0	39.0	6.6	45.1	4.4
3.7.3b	*Hin*d111	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4
	Tn*5* site:	plasmid 'd'		plasmid 'd'		plasmid 'd'		megaplasmid		chromosome		plasmid 'd'		chromosome		plasmid 'd'		chromosome	
	Plasmid Profile Class:	I		III		III		II		III		I		II		III		II	

Table 3.7.2: Summary of sizes of restriction fragments hybridising to Tn*5*-probe on Southern blots shown in Figures 3.7.4 to 3.7.6

	EXCONJUGANT:	DH119		DH120		DH122		DH124		DH125		DH126		DH201		DH204		DH205	
Figure	Enzyme	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb
3.7.4b	*Eco*R1	27.3	23.1	32.2	9.4	27.3	23.1	27.3	23.1	27.3	23.1	32.1	9.6	27.3	23.1	29.3	14.8	27.3	23.1
3.7.5b	*Bam*H1	28.5	20.3	30.3	14.1	28.5	20.3	28.5	20.3	28.5	20.3	38.8	5.6	28.5	20.3	32.2	10.2	28.5	20.3
		36.3	6.6	48.2	3.3	36.3	6.6	36.3	6.6	36.3	6.6	50.0	3.0	36.3	6.6	39.3	5.4	36.3	6.6
3.7.6b	*Hin*d111	45.5	3.4	45.5	3.4	45.5	3.4	45.5	3.4	45.5	3.4	45.5	3.4	45.5	3.4	45.5	3.4	45.5	3.4
	Tn*5* site:	plasmid 'd'		plasmid 'd'		plasmid 'c'		plasmid 'd'		plasmid 'd'		plasmid 'c'		plasmid 'd'		chromosome		plasmid 'd'	
	Plasmid Profile Class:	I		III		III		III		III		II		I		III		III	

Table 3.7.3: Summary of sizes of restriction fragments hybridising to Tn*5*-probe on Southern blots shown in Figures 3.7.7 to 3.7.9

	EXCONJUGANT:	DH209		DH211		DH212		DH213		DH214		DH216		DH221		DH222		DH225	
Figure	Enzyme	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb	mm	kb
3.7.7b	*Eco*R1	27.8	15.5	29.5	10.3	26.5	23.1	26.5	23.1	31.7	7.9	26.5	23.1	28.0	14.9	27.7	16.0	26.7	21.7
3.7.8b	*Bam*H1	46.1	5.6	37.5	11.2	34.7	20.0	34.7	20.0	40.0	6.9	34.7	20.0	34.8	19.3	36.7	12.9	35.2	18.3
		48.0	4.8	56.0	2.9	42.8	6.6	42.8	6.6	47.0	4.1	42.8	6.6	41.0	8.0	43.1	6.9	37.8	10.7
3.7.9b	*Hin*d111	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4	45.0	3.4
	Tn*5* site:	chromosome		chromosome		plasmid 'd'		plasmid 'd'		chromosome		plasmid 'd'		chromosome		chromosome		plasmid 'a'	
	Plasmid Profile Class:	III		III		III		I		III		III		II		III		II	

3.8 Investigation of the RP4-*mob*::Tn*5* Insertion Site on pOR168d

The slot-blot analysis of exconjugant genomic DNA probed with RP4-*mob* fragment revealed 62% of the screened exconjugants contained DNA homologous to the RP4-*mob* fragment. This observation was totally unexpected. The slot-blot analysis also excluded general recombination as the prime mechanism producing the observed alterations in plasmid mobility, on the basis of a lack of sequence homology between OR168 and pSUP1011. Following on from the initial findings, the aim of this section was to investigate the physical location of the RP4-*mob* fragment within the exconjugant genome, and more specifically, in pOR168d. This is to determine if the RP4-*mob* fragment has been inserted along with the Tn*5* in the pOR168d plasmid. This may lead to some indication of the mechanism involved in the insertion of the RP4-*mob* fragment.

A physical map of pSUP1011 shows that the 5.8-kb Tn*5* transposon is located within the still functional 2.6-kb RP4-derived *mob* fragment (Simon *et al.*, 1983a). Because the Tn*5* is carried with the *mob* fragment, the potential for the RP4-derived sequence to be transposed along with Tn*5* (due to error?) by the transposition mechanism needs to be investigated.

Three exconjugants, DH113, DH201 and DH216, carrying both Tn*5* and the RP4-*mob* fragment on pOR168d, were selected to determine if both genetic elements are together on the same DNA fragment, as they are in the vector. The exconjugants were also chosen to characterise more precisely the site of insertion of the pSUP1011-derived DNA. The three are analogous exconjugants in that Tn*5* occupies approximately the same site in all three exconjugants (see Figures 3.7.1 to 3.7.9).

The plasmid profiles of DH113 and DH201 represent the predominant pattern seen in the screened exconjugants (both Class I exconjugants), with one example originating from each cross. Tn*5* is located on the 40-kb 'd' plasmid for both. By choosing examples with the same plasmid profile from the two crosses, it is hoped to determine if the observed pattern is derived via the same mechanism. The plasmid profile of the other exconjugant, DH216, is unique in that it is the only exconjugant exhibiting that plasmid profile. DH216 contains Tn*5* on the 65-kb pDH216d plasmid. With that being stated, the only difference in the plasmid profile of the three is the size of plasmid 'd'.

After extracting total genomic DNA from the exconjugants, combinations of single and double restriction endonuclease digests were carried out as listed in Table 3.8.1. In each digest reaction the conditions used ensured complete cleavage of the DNA

(see Section 2.10.2 for details). The restriction fragments produced were separated using conventional gel electrophoresis. Figure 3.8.1a shows the gel from which a Southern blot was prepared for probing with DNA sequences specific for Tn*5* and RP4-*mob*. Detection of hybridising gel fragments was carried out using the Chemiluminescent system developed by Boehringer Mannheim GmbH with DIG-labelled probes. For details of the exact methodology refer to Section 2.12. The stripping and reprobing of the same Southern blots facilitated comparisons of hybridisation patterns. The addition of DNA size markers labelled with DIG on the gels made direct estimation of the band sizes possible.

The locations of the DNA sequences of interest, RP4-*mob* and Tn*5*, were determined by Southern hybridisation using the specific DNA probes from pSUP202, pKan2 and pSUP1011. By using these overlapping probe sequences in conjunction with a series of single and double restriction enzyme digests allows for the construction of restriction maps around the insertion site of the donor DNA. It is clear from the hybridisation patterns observed for each exconjugant on the lumigraphs seen in Figures 3.8.1b to 3.8.1d that the location of RP4-*mob* and Tn*5* is the same fragment, and that the site of insertion in the three exconjugants is identical. A list of the sizes of the fragments detected by Southern hybridisation is shown in Table 3.8.1. These data were used to construct restriction maps of the Tn*5*/RP4-*mob* insertion sites on plasmid 'd' for the three exconjugants studied. The restriction maps for each of the exconjugants are shown in Figure 3.8.2.

The only difference in the restriction maps for each exconjugant was the size of the large *Eco*RI fragment (shown diagrammatically on the right). The size differs from the results obtained in Section 3.7.1 for the sizes of the restriction fragments hybridising to the Tn*5* probe. This discrepancy between the two sets of results has probably arisen from a combination of limitations in the electrophoresis when sizing bands greater than 23.1 kb when the largest standard band is only 23.1 kb, and extrapolation errors when estimating band size on semi-$\log_{10}$ plots of plasmid size against distance migrated.

The pSUP202-derived probe with the shorter RP4 DNA sequence revealed a single 11.3 kb *Eco*RI/*Hin*dIII fragment homologous to the RP4-*mob* fragment. The location of the RP4-*mob* fragment is bounded by the same *Eco*RI and *Hin*dIII restriction sites for the three exconjugants. By deduction, the 6.0 kb *Hin*dIII/*Bam*HI fragment with homology to pSUP1011 is the location of the rest of the RP4-*mob* fragment not present in pSUP202. Although the orientation of Tn*5* cannot be ascertained from the Southern blots with the probes used, it is clear that both the Tn*5* and RP4-derived DNA have the same physical location relative to each other as in the pSUP1011

plasmid. This suggests that both donor sequences have moved from pSUP1011 at the same time in a contiguous block.

Although the 27.6 kb defined by the *Hin*dIII sites and containing the Tn*5*::RP4-*mob* fragment appears to be identical in DH113, DH201 and DH216, the clear difference in the location of the *Eco*RI site to the right in these three plasmids is puzzling. In the absence of further work, we can only presume that the observed differences at a greater distance are a reflection of a rearrangement event, perhaps of the sort that generated the diversity of plasmid profiles.

Figure 3.8.1: Electrophoresis and detection of pSUP1011-derived DNA sequences in digested total genomic DNA from the exconjugants DH113, DH201 and DH216.

Figure 3.8.1a. Agarose gel electrophoresis separation of single and double restriction fragments of total genomic DNA digested using the restriction enzymes *Bam*HI, *Eco*RI, and *Hin*dIII. DNA preparation and digestion, electrophoresis, and gel staining were performed as described in Materials and Methods section.

DNA source:	**Lane #:**	**Restriction enzymes used:**
DH113	1	*Eco*RI
	2	*Bam*HI
	3	*Hin*dIII
	4	*Eco*RI/*Bam*HI
	5	*Eco*RI/*Hin*dIII
	6	*Bam*HI/*Hin*dIII
DH201	7	*Eco*RI
	8	*Bam*HI
	9	*Hin*dIII
	10	*Eco*RI/*Bam*HI
	11	*Eco*RI/*Hin*dIII
	12	*Bam*HI/*Hin*dIII
DH216	13	*Eco*RI
	14	*Bam*HI
	15	*Hin*dIII
	16	*Eco*RI/*Bam*HI
	17	*Eco*RI/*Hin*dIII
	18	*Bam*HI/*Hin*dIII
OR168	19	*Eco*RI
DIG-labelled λ	20	*Hin*dIII λDNA size markers

Figure 3.8.1a: Gel electrophoresis of *Eco*RI, *Bam*HI, and *Hin*dIII restriction fragments of total genomic DNA isolated from DH113, DH201, and DH216

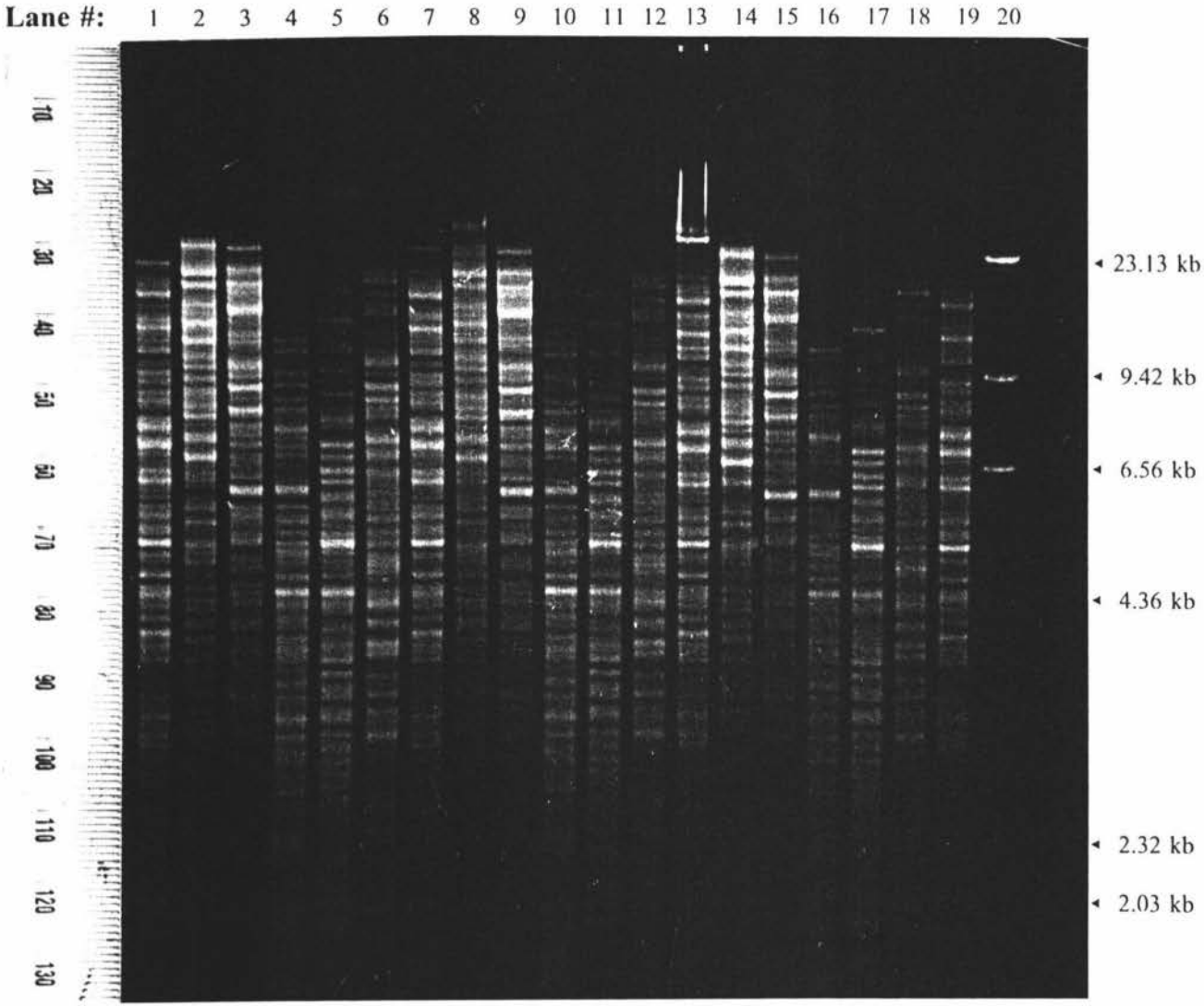


Figure 3.8.1 - continued:
Electrophoresis and detection of pSUP1011-derived DNA sequences in digested total genomic DNA from the exconjugants DH113, DH201 and DH216.

Figure 3.8.1b. Southern hybridisation of the gel shown above, in Fig. 3.8.1a, probed with the internal *Hin*dIII fragment from pKan2 to identify the fragments containing the Tn*5* element. Hybridisation and detection steps were performed as described in the Materials and Methods section, with OR168 DNA being used as an internal negative control.

DNA source:	**Lane #:**	**Restriction enzymes used:**
DH113	1	*Eco*RI
	2	*Bam*HI
	3	*Hin*dIII
	4	*Eco*RI/*Bam*HI
	5	*Eco*RI/*Hin*dIII
	6	*Bam*HI/*Hin*dIII
DH201	7	*Eco*RI
	8	*Bam*HI
	9	*Hin*dIII
	10	*Eco*RI/*Bam*HI
	11	*Eco*RI/*Hin*dIII
	12	*Bam*HI/*Hin*dIII
DH216	13	*Eco*RI
	14	*Bam*HI
	15	*Hin*dIII
	16	*Eco*RI/*Bam*HI
	17	*Eco*RI/*Hin*dIII
	18	*Bam*HI/*Hin*dIII
OR168	19	*Eco*RI
DIG-labelled λ	20	*Hin*dIII λDNA size markers

Figure 3.8.1b: Southern blot of gel in Fig 3.8.1a probed with the 3.5-kb *Hin*dIII fragment of pKan2 (Tn*5*)

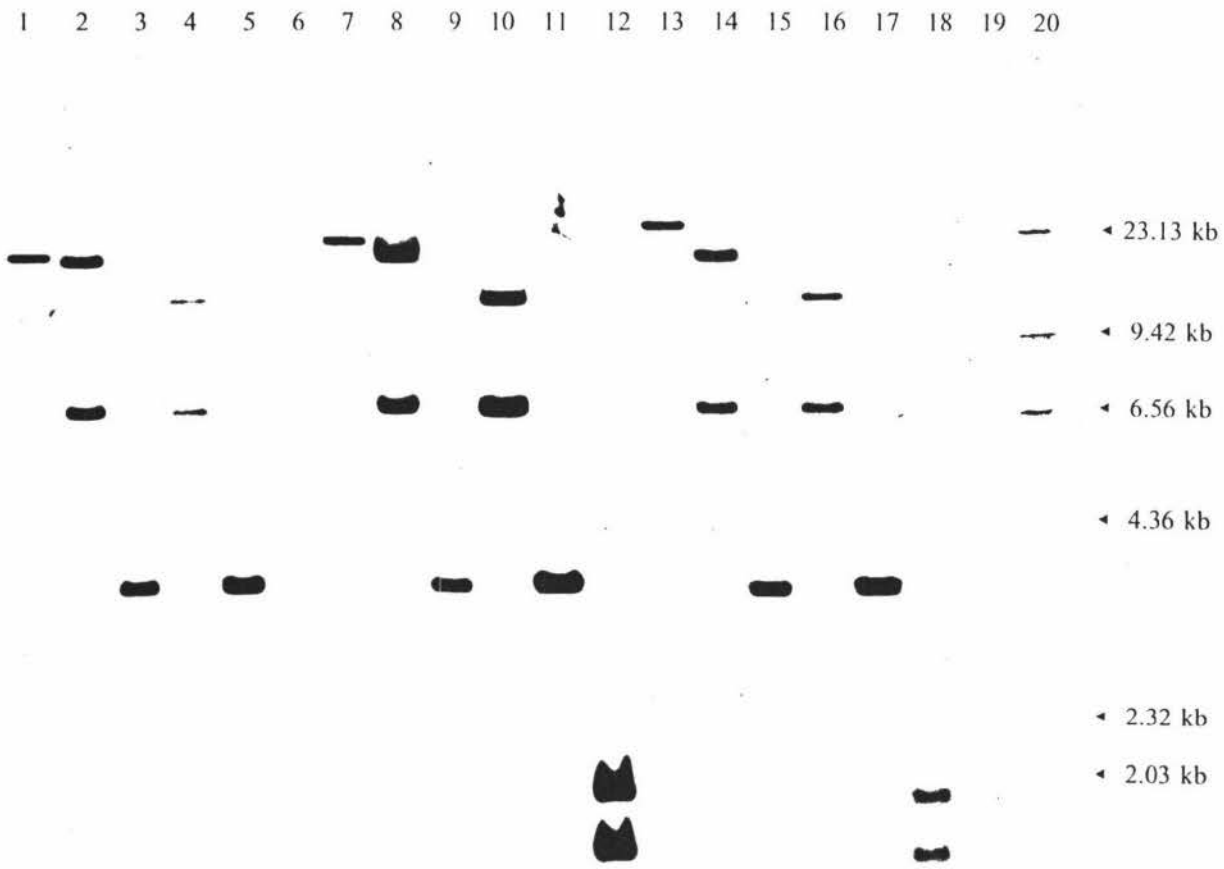


Figure 3.8.1 - continued:
Electrophoresis and detection of pSUP1011-derived DNA sequences in digested total genomic DNA from the exconjugants DH113, DH201 and DH216.

Figure 3.8.1c. Southern hybridisation of the gel shown above, in Fig. 3.8.1a, stripped and reprobed with the DIG-labelled 3.1-kb *Eco*RI/*Hin*dIII fragment from pSUP202 to identify the fragments with homology to the 1.9-kb RP4-derived *oriT*-containing fragment.

DNA source:	**Lane #:**	**Restriction enzymes used:**
DH113	1	*Eco*RI
	2	*Bam*HI
	3	*Hin*dIII
	4	*Eco*RI/*Bam*HI
	5	*Eco*RI/*Hin*dIII
	6	*Bam*HI/*Hin*dIII
DH201	7	*Eco*RI
	8	*Bam*HI
	9	*Hin*dIII
	10	*Eco*RI/*Bam*HI
	11	*Eco*RI/*Hin*dIII
	12	*Bam*HI/*Hin*dIII
DH216	13	*Eco*RI
	14	*Bam*HI
	15	*Hin*dIII
	16	*Eco*RI/*Bam*HI
	17	*Eco*RI/*Hin*dIII
	18	*Bam*HI/*Hin*dIII
OR168	19	*Eco*RI
DIG-labelled λ	20	*Hin*dIII λDNA size markers

Figure 3.8.1c: Southern blot of gel in Fig 3.8.1a stripped and reprobed with the *Eco*R1/*Hin*d111 fragment of pSUP202 (1.9-kb RP4-*mob* fragment)

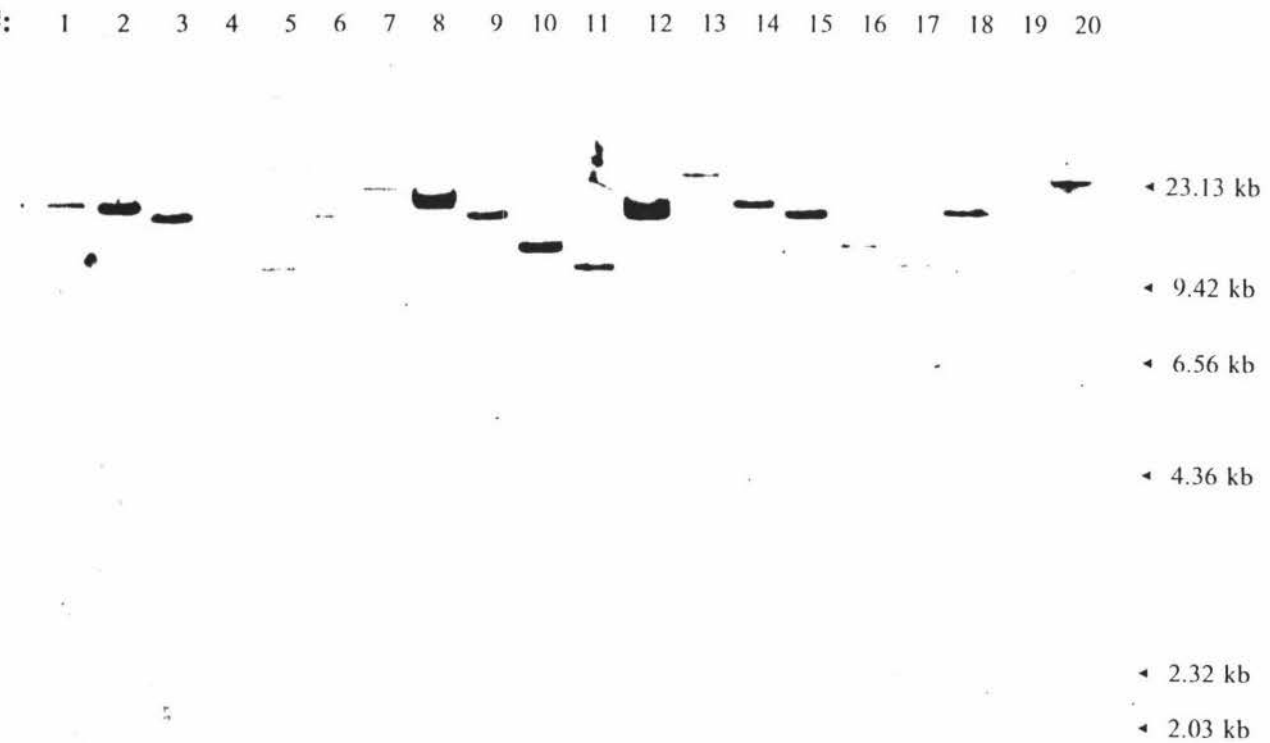


Figure 3.8.1 - continued: Electrophoresis and detection of pSUP1011-derived DNA sequences in digested total genomic DNA from the exconjugants DH113, DH201 and DH216.

Figure 3.8.1d. Southern hybridisation of the gel shown in Fig. 3.8.1a, stripped and reprobed with the entire DIG-labelled pSUP1011 vector, to identify all fragments containing DNA homologous to pSUP1011, namely Tn*5*, the 2.6-kb RP4-derived *oriT*-containing fragment, and pACYC184.

Probe stripping, rehybridisation and detection steps were performed as described in the Materials and Methods section. OR168 DNA was used as an internal negative control.

DNA source:	**Lane #:**	**Restriction enzymes used:**
DH113	1	*Eco*RI
	2	*Bam*HI
	3	*Hin*dIII
	4	*Eco*RI/*Bam*HI
	5	*Eco*RI/*Hin*dIII
	6	*Bam*HI/*Hin*dIII
DH201	7	*Eco*RI
	8	*Bam*HI
	9	*Hin*dIII
	10	*Eco*RI/*Bam*HI
	11	*Eco*RI/*Hin*dIII
	12	*Bam*HI/*Hin*dIII
DH216	13	*Eco*RI
	14	*Bam*HI
	15	*Hin*dIII
	16	*Eco*RI/*Bam*HI
	17	*Eco*RI/*Hin*dIII
	18	*Bam*HI/*Hin*dIII
OR168	19	*Eco*RI
DIG-labelled λ	20	*Hin*dIII λDNA size markers

Figure 3.8.1d: Southern blot of gel in Fig 3.8.1a stripped and reprobed with pSUP1011 (2.6-kb RP4-*mob* fragment + Tn*5*)

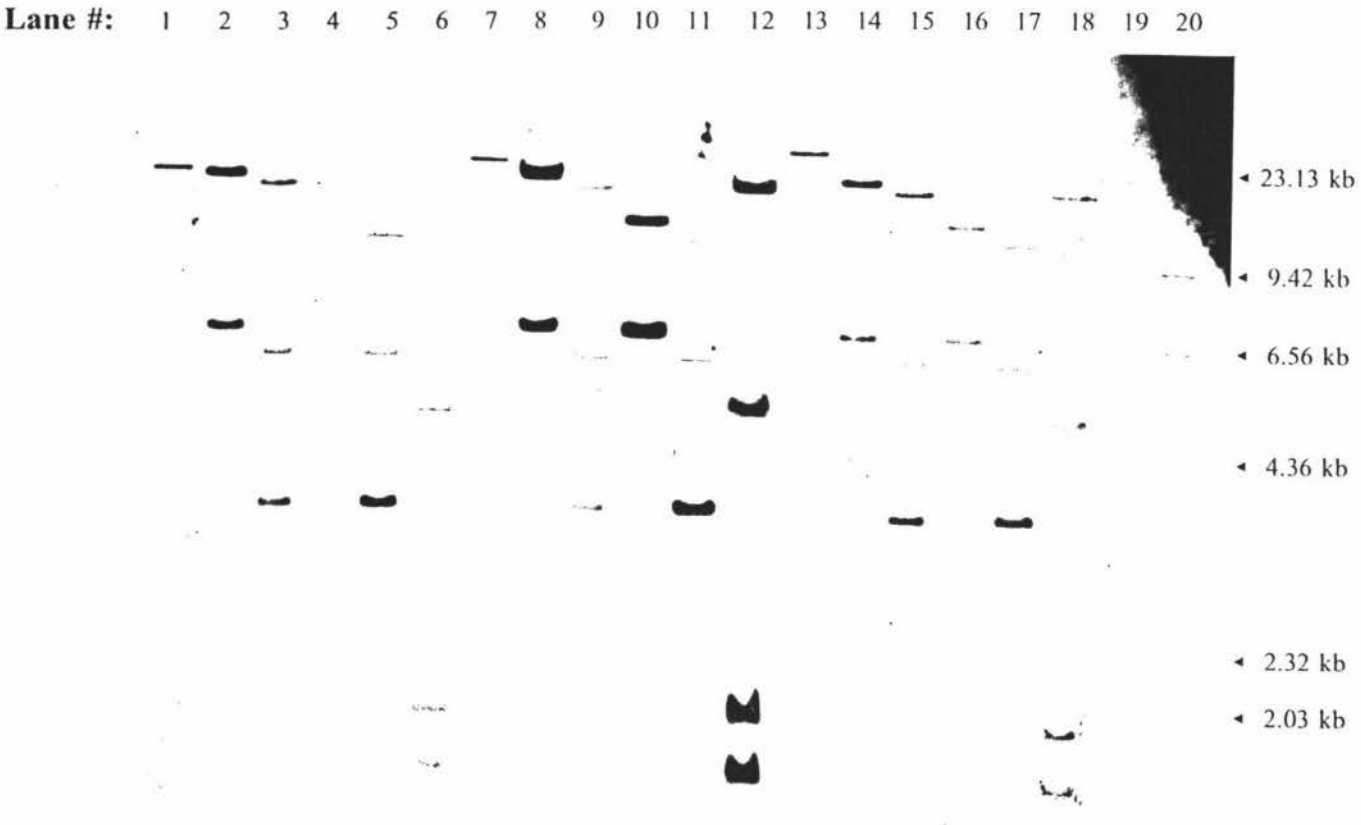


TABLE 3.8.1: Summary of total genomic DNA fragment sizes hybridising to probes derived from pKan2, pSUP202, and pSUP1011.

DNA source:	Lane #	Restriction enzymes:	Size (kb) of Fragments Hybridising to the following probe sequences:- pKan2†:		pSUP202‡:	pSUP1011*:			
			Band #: 1	2	1	1	2	3	4
DH113	1	*Eco*RI	22.0		22.0	22.0			
	2	*Bam*HI	20.0	6.6	20.0	20.0	6.6		
	3	*Hin*dIII	3.4		17.8	17.8	6.0	3.4	
	4	*Eco*RI/*Bam*HI	13.2	6.6	13.2	13.2	6.6		
	5	*Eco*RI/*Hin*dIII	3.4		11.3	11.3	6.0	3.4	
	6	*Bam*HI/*Hin*dIII	1.9	1.6	17.8	17.8	5.0	1.9	1.6
DH201	7	*Eco*RI	24.3		24.3	24.3			
	8	*Bam*HI	20.0	6.6	20.0	20.0	6.6		
	9	*Hin*dIII	3.4		17.8	17.8	6.0	3.4	
	10	*Eco*RI/*Bam*HI	13.2	6.6	13.2	13.2	6.6		
	11	*Eco*RI/*Hin*dIII	3.4		11.3	11.3	6.0	3.4	
	12	*Bam*HI/*Hin*dIII	1.9	1.6	17.8	17.8	5.0	1.9	1.6
DH216	13	*Eco*RI	28.2		28.2	28.2			
	14	*Bam*HI	20.0	6.6	20.0	20.0	6.6		
	15	*Hin*dIII	3.4		17.8	17.8	6.0	3.4	
	16	*Eco*RI/*Bam*HI	13.2	6.6	13.2	13.2	6.6		
	17	*Eco*RI/*Hin*dIII	3.4		11.3	11.3	6.0	3.4	
	18	*Bam*HI/*Hin*dIII	1.9	1.6	17.8	17.8	5.0	1.9	1.6

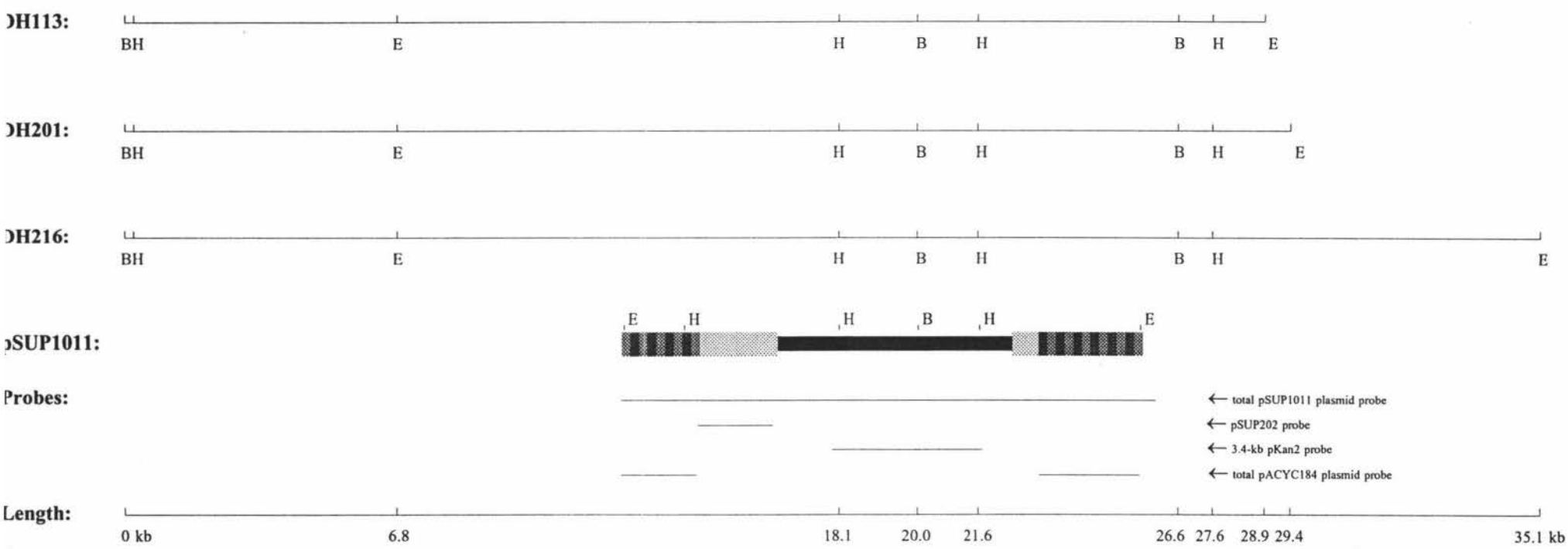
NOTE: Data obtained from analyses of lumigraphs shown in Figure 3.8.1.

† pKan2 probe is the 3.4 kb internal *Hin*dIII fragment of Tn*5*.

‡ pSUP202 probe is the 3.1 kb *Eco*RI/*Hin*dIII fragment from pSUP202 containing a 1.9-kb RP4-*oriT* fragment.

* pSUP1011 probe is the entire pSUP1011 plasmid containing a complete Tn*5* plus a 2.6-kb RP4-*oriT* fragment.

Figure 3.8.2: Restriction mapping of the insertion site of the donor DNA sequences in plasmid 'd' of exconjugants DH113, DH201 and DH216



LEGEND: Restriction sites: B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII

2.6-kb RP4-derived sequence containing the *oriT* site:

5.8-kb Transposon Tn*5* sequence:

4.2-kb pACYC sequence:

The schematic diagram above shows the relative position of the three probe sequences used to locate donor DNA sequences from pSUP1011 within the three exconjugants, DH113, DH201, and DH216. The diagram is drawn to scale to show the relationship between the vector pSUP1011 and the exconjugants. The pACYC sequence present in pSUP1011 was not used as a probe in the restriction mapping because previous slot-blot hybridisation revealed the absence of this sequence in the exconjugants. It's position is indicated in the schematic as a reference only (also refer to Table 3.6.1).

3.9 Plasmid Rearrangements Revisited

More information is now available since the initial analysis was made in Section 3.3 regarding the observed plasmid rearrangements of the exconjugant population following the introduction of the transposon Tn*5*. Further analysis is restricted by the lack of plasmid-specific probes. Without them the altered plasmids observed in the exconjugants cannot be positively identified. It must also be realised that the identity of the rearranged plasmids is based purely on conjecture, with their identity generally based on the comparative presence or absence of particular bands on the plasmid profiles obtained from Eckhardt gel electrophoresis.

This section is to serve basically as a retrospective summary of information obtained concerning the investigation of the cause and effect for the observed plasmid rearrangements after the conjugative introduction of pSUP1011 into the OR168 genome.

Since the initial comparison of plasmid profiles in Section 3.3, where it was shown that no exconjugant has the OR168 plasmid profile, it has been shown by total genomic restriction analysis that all exconjugants have a banding pattern equivalent to the OR168 parent. This proves the common ancestry of the exconjugant population and that the exconjugants are not isolated contaminants.

Plasmid 'm' of OR168, the megaplasmid, is generally present in all exconjugants. The transposon Tn*5* has been detected in this plasmid in only two exconjugants, and it could be argued that this was only by fortuitous chance. It is assumed that because of its large size and presumably low copy number (which will result in low target concentrations for the Tn*5* probe), the presence of Tn*5* in this replicon may not have always been detected. Even so, the mobility of this plasmid appears stable, with only one exconjugant (DH106) showing any significant alteration in its mobility. But because of its size, any alteration would have to be significant to be detected on the Eckhardt gels.

The mobility of plasmid 'a' of OR168 appears unaltered and present in all the exconjugants. The detection of no discernable alterations in its mobility could be attributed to the relatively large size of this plasmid. It was the site for Tn*5* insertion in one case (DH225), without any measurable change in mobility.

Plasmid 'b' of OR168 is always rearranged in the exconjugant population, but never the site of insertion of any pSUP1011-derived sequences. Generally, the detectable change in mobility equates to the insertion of 70 kb. But there are examples where

the change in mobility equates to insertions of well in excess of 100 kb. Conversely, there are changes in mobility for this plasmid equating to deletions of well in excess of 100 kb. The exconjugant DH214 shows no plasmid corresponding to plasmid 'b'. The cause and structural basis of these changes are unknown.

Plasmid 'c' of OR168 shows alteration in mobility in only three exconjugants. The change in mobility of the plasmid 'c' in DH122 coincides with the insertion of the RP4-*mob*::Tn*5* fragment from pSUP1011. But the estimated size of this fragment is 8.4 kb, while the alteration in mobility of pDH122c equates to the insertion of 37 kb. The other two exconjugants showing altered mobility to plasmid 'c', DH116 and DH211, both have changes that suggest deletions of 43 kb. These changes have both coincided with the addition of an estimated 100 kb to plasmid 'b' for both exconjugants. A further perplexing observation is the loss of this plasmid from DH209 when its plasmid profile was rechecked using Eckhardt gel electrophoresis (compare Fig 3.3.4, Lane # 9 with Fig 3.3.9, Lane # 1). This is the only observed further alteration to the plasmid profile after repeated subculture of the exconjugants. All other exconjugants and the parent, OR168, appear stable.

Plasmid 'd' is one of the most frequently altered plasmids, with 60% of the screened exconjugant population showing altered mobility in this plasmid. In only one case can the alteration be attributed solely to the insertion of Tn*5*. This occurred in DH120 (see Fig 3.3.3, Lane #1). The alteration in mobility to plasmid 'd' generally appears to equate to the addition of 40 kb of DNA. Again, this change in mobility coincides with the insertion of the RP4-*mob*::Tn*5* fragment from pSUP1011.

From the Tn*5* probing of genomic restriction fragments it has been shown that universally, there is only a single copy of the transposon in the exconjugant genomes. The presence of a single Tn*5* element eliminates the possibility of recombination between repeated Tn*5* elements as a basis for plasmid rearrangement by means of non-reciprocal deletions or duplications of the intervening DNA.

But precise excision of Tn*5* can also occur at low frequencies (Berg *et al.*, 1983a). This excision of Tn*5* does not appear to be caused by any known endonuclease, but the short direct repeats are known to serve as hot spots for spontaneous deletion formation (Collins *et al.*, 1982). A possible scenario for events causing the observed plasmid rearrangements could involve multiple insertions/transpositions followed by general recombination between Tn*5* elements. This in turn results in deletions and insertions, followed by precise excision and the loss of Tn*5*. Because this scenario involves the occurrence of a number of independent but sequentially dependent events their probability of actually happening is greatly reduced. Furthermore, many of the events appear to occur repeatedly, yielding identically altered plasmids.

3.10 Transmissible Plasmids in OR168::Tn*5* Exconjugants

Taken together, the slot-blot hybridisation results summarised in Table 3.6.1 and the Southern blot results summarised in Table 3.8.1, reveal the concomitant insertion of the RP4-specific *mob* fragment with plasmid-borne Tn*5* elements for the exconjugants DH113, DH201, and DH216. This is probably a wide-spread phenomenon, occurring in each OR168::Tn*5* exconjugant with the RP4-*mob* fragment. An *oriT* site is essential for the conjugative transfer of plasmids since it provides the *cis*-acting *nic/bom* site. For the plasmid pSUP1011, the RP4-specific *oriT* site is contained within the *mob* fragment. Assuming there are RP4-specific *trans*-acting factors present in the OR168::Tn*5* exconjugants, the presence of the RP4 *mob* site on the exconjugant plasmid containing Tn*5*, creates the possibility that the plasmid is mobilisable as well as selectable.

3.10.1 Attempted Recovery of OR168::RP4-*oriT*::Tn*5* Plasmids

The opportune recovery of the Tn*5*-bearing plasmid DNA via conjugative transfer into a plasmid-free host would provide plasmid material ideal for use as a source of probe DNA for that specific plasmid. Recovery of Tn*5*-bearing plasmids would also provide material for more detailed studies of the RP4::Tn*5* insertional 'hot spot' on plasmid pOR168d. Initially exconjugants were screened to see if any of the Tn*5*-bearing plasmids were self-mobilisable. It is unknown whether OR168::Tn*5* plasmids can be stably transferred into a host lacking Tn*5*. Because the recipient host lacks a resident Tn*5*, it also lacks the *trans*-acting protein product of IS*50*R, which suppresses transposition of Tn*5*. Consequently, even if transfer was achieved, conservative transposition could result in the loss of the transferred RP4::Tn*5* plasmid.

Repeated attempts to conjugatively transfer plasmids from OR168::Tn*5* exconjugants into both a plasmid-free, streptomycin-resistant *E. coli* HB101 strain and a Tn*5*-free, rifampicin-resistant OR168-derivative failed to yield any neomycin-resistant isolates. This showed that the Tn*5*-bearing plasmids were not self transmissible. Due to time constraints, no further attempt was made to isolate the Tn*5*-bearing plasmids from OR168::Tn*5* exconjugants. This may have been easily achieved via triparental matings using a helper plasmid, such as pRK2013 (Figurski & Helinski, 1979). This narrow host-range vector could provide the essential IncP-specific *trans*-acting factors for the conjugative transfer of the RP4-*mob*::Tn*5*-bearing plasmids from OR168::Tn*5* exconjugants.

Attempts to recover RP4-*mob*::Tn*5*-bearing plasmids using CsCl/EtBr gradient centrifugation, following initial plasmid isolation protocols from Casse *et al* (1979), and Hayman & Farrand (1990) also failed. This highlights the need to optimise the conditions for plasmid extraction from OR168 and its exconjugants.

4. GENERAL DISCUSSION AND CONCLUSIONS

4.1 Discussion

4.1.1 The Relationship Between the Site of Tn*5* Insertion and Plasmid Rearrangement

Tn*5* insertion mutants of OR168 were easily obtained using conjugative transfer of the Tn*5*-bearing suicide plasmid, pSUP1011. From plasmid profile analysis of the OR168::Tn*5* exconjugants (refer to Figure 3.5.1), there appears to be no direct relationship between the site of Tn*5* insertion and plasmid rearrangement. There are instances of both non-Tn*5*-bearing and Tn*5*-bearing plasmids being rearranged within the same exconjugant. There is also plasmid rearrangement in some exconjugants without any evidence of plasmid-borne Tn*5* elements. As with the plasmid profile of the parental strain, OR168, the plasmid profile exhibited by each exconjugant studied appears stable, with no further rearrangements seen after repeated subculture under laboratory conditions, which suggests the observed rearrangements are stable.

The rearrangements appear to be triggered by the introduction of pSUP1011, the Tn*5*-bearing plasmid, into the genome. The dynamics of the recombination events are difficult to explain because only limited 'before' and 'after' views can be seen. Another factor limiting our understanding of the observed rearrangements is the narrow selection of recombination products. Many theoretical products of Tn*5* transposition and recombination may not be viable in the OR168 host. This is particularly true of events involving excision or loss of Tn*5*, since there was continued selective pressure to isolate only Tn*5*-bearing exconjugants.

No work has been done on studying the recombination pathways of OR168, so any explanation for the observed events can only be modelled on similar events seen in other Gram-negative eubacteria following the introduction of Tn*5*. In each case, the rearrangements appear to involve either the deletion or insertion of large sequences of DNA, even when there appears to be no DNA sequence homology between the OR168 recipient and the mobilisable pSUP1011 plasmid. This rules out general recombination as the primary mechanism responsible for the alterations in plasmid profile. The results do suggest the involvement of other mechanisms such as illegitimate recombination or the combined result of different recombination mechanisms acting in concert, such as site-specific recombination events followed by

general recombination; or some previously uncharacterised activity of the transposase enzyme.

4.1.2 Insertion of Tn*5* with and without the RP4-*mob* fragment into Plasmid pOR168d

The 22-kb pOR168d plasmid appears to be the preferred site of Tn*5* insertion among the 57 OR168::Tn*5* exconjugants screened (See Figure 3.5.1 for a summary of comparative plasmid profiles). All exconjugants showing an alteration in the mobility of pOR168d carry the Tn*5* on that plasmid. But the insertion of DNA into pOR168d from pSUP1011 has only partially contributed to the observed alteration in its mobility. From the plasmid sizing exercise undertaken in Section 3.2, the typical shift in mobility equates to an insertion of approximately 18 kb, which is 3 times greater than that expected from the 'transpositional' insertion of the 5.8 kb Tn*5* alone. This mobility shift equates to a change in size from 22 kb to 40 kb. Of the 57 exconjugants screened, 29 have acquired this 40-kb plasmid 'd', and all 29 carry the Tn*5* on this plasmid. The simple transposition of Tn*5* into the pOR168d plasmid is not sufficient to cause the large shift in mobility seen for this plasmid in the Eckhardt gels shown in Figures 3.3.1 to 3.3.9. Nor is the observed alteration in the mobility of pOR168d invariable. For DH216 (Fig 3.3.5, Lane # 2) the insertion of Tn*5* into pOR168d has resulted in a change in mobility equating to the addition of 43 kb of DNA. For DH125 (Fig 3.3.2, Lane # 7), the Tn*5* insertion into pOR168d equates to the addition of 14 kb of DNA. But for DH120 (Fig. 3.3.3, Lane # 1), the insertion of Tn*5* into the 22-kb plasmid has resulted in a change in mobility equating to an insertion of 6 kb only, which suggests a true conservative transposition event.

Slot-blot analysis of total genomic DNA from the exconjugants that was probed for the presence of pSUP1011 DNA sequences (refer to Table 3.6.1), showed the presence of the RP4-derived *mob*-containing DNA fragment in every exconjugant carrying a presumptive pOR168d of altered mobility in excess of approximately 6 kb. Furthermore, Southern probing of the Eckhardt gel, shown in Figure 3.3.7, for the presence of both Tn*5* and the RP4-*mob* fragment, has revealed that when the RP4-*mob* fragment is present, it is contained on the same replicon as the Tn*5*.

Restriction mapping of the three exconjugants, DH113, DH201, and DH216 has revealed that Tn*5* and the RP4-*mob* fragment are situated within the same *Eco*RI fragment. This result shows, unambiguously, the simultaneous insertion of Tn*5* and RP4-*mob* into pOR168d in these exconjugants, although the mechanism of the insertion is not understood. Does this suggest that the insertion of the RP4-*mob*::Tn*5* fragment within the pOR168d plasmid involves an insertional 'hot spot'?

From analysis of the restriction fragments highlighted by the probing for pSUP1011 sequences (see Fig 3.8.2), there is only presumptive evidence that, at the site of insertion, the transposon is bordered by the RP4-*mob* fragment at both (or at least one) OE sequences. It shows that these three exconjugants have not acquired Tn*5* as a result of conventional conservative transposition. Without further investigation the actual mechanism for the RP4-*mob* fragment transfer from pSUP1011 can only be speculated. It is also only an assumption that the other 26 exconjugants with Tn*5* and RP4-*mob* DNA in plasmid 'd' have acquired this DNA by the same mechanism.

The results from the restriction mapping exercise shown in Figure 3.8.2 and Table 3.8.1, show the same insertion site within pOR168d for the RP4-*mob*::Tn*5* fragment as defined by the *Hin*dIII sites for the three exconjugants analysed. The mapping of the site into which the Tn*5* has inserted on pOR168d has also revealed different lengths for the large *Eco*RI fragment in each of the three exconjugants examined. This is shown in Figures 3.7.1 (lane 6), 3.7.4 (lane 7), and 3.7.7 (lane 6). Closer examination suggests that the slight variations in band mobility that were initially attributed purely to variations in the electrophoretic conditions, may actually be physical differences of size. Given that these size differences are relatively small, in comparison to the size of the DNA fragment with which they are associated, and the attendant uncertainty of accurately sizing any fragments greater than 20 kb on the blots, no significant conclusions can be drawn from this observation without further investigation. The positions of the three *Eco*RI bands on the Southern blot in Figure 3.8.1 clearly show that they are significantly different, and this is not due to electrophoretic variability.

Assuming the total insertion of the 2.6-kb RP4-*mob* fragment in the exconjugants where it has been detected, then the origin of only 8.4 kb of the extra 18 kb of DNA in the altered 40-kb pOR168d can be accounted for (5.8 kb for Tn*5* plus 2.6 kb for RP4-*mob*).

By comparison, the mobility of the 163-kb pOR168c plasmid has been altered in only three OR168::Tn*5* exconjugants isolated. These changes in mobility equate to deletions of 43 kb for DH116 and 63 kb for DH211 and an insertion of 37 kb for DH122. The change in mobility of pDH122c is associated with the insertion of Tn*5*, along with the presumed acquisition of the RP4-*mob* fragment which, from the slot-blot analysis, is also present in this exconjugant. The pOR168c plasmid is the recipient of Tn*5* in only two of the exconjugants screened, namely DH122 and DH126 (see Figure 3.3.8). The former appears to be another example of an anomalous RP4-*mob*-bearing insertion (Table 3.6.1), whereas the latter appears to be a conventional Tn*5* insertion. This conclusion is confirmed by comparing the sizes of the restriction fragments hybridising to the Tn*5*-probe DNA for both DH122 and

DH126 (see Table 3.7.2). Their different sizes infer different sites of insertion for the Tn*5*, although the fragments observed for DH122 are identical to those observed for the RP4-*mob*-bearing plasmid 'd' inserts. This observation suggests that the site of anomalous insertion is both extensive and found in both plasmids pOR168 and pOR168c.

The mobility of the 225-kb pOR168b plasmid has been altered in all the exconjugants isolated. The alterations cannot be explained as a result of transposition, nor as a result of recombination events involving the pSUP1011 vector. The altered plasmid does not appear to be a site for Tn*5* insertion at all. The observed change in mobility of pOR168b in the exconjugants equates to estimated changes in plasmid size ranging from the addition of 170 kb for DH116 and DH211, to the deletion of 142 kb for DH204. Extraordinarily, in DH106, the size of the altered 'b' plasmid is estimated to be 600 kb, indicating a doubling in size. For the exconjugant DH214 there appears to be no plasmid present equivalent to pOR168b, with it only showing three plasmid bands. Together, the results suggest events other than transposition are causing extensive rearrangements to this plasmid.

The mobility of the 570-kb pOR168a plasmid appeared stable, showing no detectable change, even with the insertion of Tn*5*. A single OR168::Tn*5* exconjugant (DH225) showed insertion of the Tn*5* into this plasmid and without the concomitant insertion of the RP4-*mob* fragment. This infers a true transposition event for DH225. No change in mobility was observed because the insertion of an extra 5.8 kb would not be detected in a plasmid of the size of pOR168a, with the electrophoresis system used.

It is interesting to note that even though rearrangements of some description accompany every acquisition of Tn*5*, those labelled Class I occur only when pOR168d has acquired the combined RP4-*mob*::Tn*5* fragment (see Table 3.6.1). In contrast, the Class II rearrangement occurs only in association with what appears to be 'classical' (that is, conservative) Tn*5* transpositions. However, this generalisation is not exclusive, in that both the 'classical' and the anomalous (including the RP4-*mob* fragment) transpositions can be associated with other patterns of plasmid rearrangement, such as the Class III patterns observed (see Table 3.6.1).

4.1.3 Reports of Anomalous Tn*5* Activity and their Relevance to Observations Made in this Study

One initial aim of this study was to tag plasmids native to OR168 with the transposon Tn*5* and follow their conjugative transfer to other organisms. This was planned in order to gain an insight into the processes causing alterations to plasmid profiles as observed in natural soil populations of bacteria after the introduction of a new replicon. It is thought the alterations are precipitated by recombination between homologous or reiterated sequences on the resident and introduced replicons. The introduction of the plasmid vector pSUP1011 into the genome of the soil isolate OR168 produced wide variations in the native plasmid profile. It was considered pertinent to investigate more fully these observed alterations before attempting plasmid transfer to other hosts, especially when the patterns of rearrangements observed in OR168::Tn*5* exconjugants mimicked those observed in natural populations. Although pSUP1011 is a genetically engineered suicide plasmid and shows no gross sequence homology to the OR168 genome, its presence precipitated alterations in the plasmid profile of OR168. This activity may still be solely attributable to the transposon Tn*5*, rather than an implied activity of pSUP1011. We is no evidence relevant to answering this question.

Alteration in the plasmid profiles following Tn*5* mutagenesis of *Rhizobium* has been reported in a number of studies. Meade *et al.*, (1982) used the suicide plasmid vector pJB4JI to introduce Tn*5* conjugatively into *R. meliloti* for the construction of symbiotic mutants. They reported a low frequency of plasmid rearrangement and concluded that it was due to the simultaneous transposition of both phage Mu and Tn*5* (Tn*5* is located within Mu on this vector).

Noel *et al.*, (1984) using the same suicide vector to form random symbiotic defective mutants in *R. phaseoli* reported plasmid loss as well as the formation of new plasmids which were Tn*5*-containing deleted forms of the vector pJB4JI. The Mu-induced failure of pJB4JI to replicate was overcome and the plasmid derivatives were able to stably replicate.

Transposition events tended to be overshadowed by these vector-induced events. The occurrence of such problems complicated analysis of results and shows the need to screen carefully the exconjugant population for the presence of vector sequences when using pJB4JI. The development of the pSUP-series of vectors by Simon *et al.* (1983) allowed for an alternative conjugative delivery system for Tn*5* mutagenesis. This system is thought to circumvent the anomalies incurred when using pJB4JI.

But there have been reports of anomalous transposition activity by pSUP-series vectors after transfer into a new host. A study by Tully *et al.*, (1992) investigating the suitability of different transposon mutagenesis systems for *Legionella pneumophila*, showed pSUP1021 [pACYC184 *mob* Cm^R Tn*5*, (Simon *et al.*, 1986)] was able to replicate stably in the *Legionella* recipients. Donald *et al.* (1985) reported the exclusive formation of plasmid-genome cointegrates when attempting Tn*5* mutagenesis of *Azorhizobium caulinodans* strain ORS571 using the pSUP-series derivative pVP2021 (pSUP202::Tn*5*: pBR325 *mob* Ap^R Cm^R Tc^R Tn*5*). In the current study, the slot-blot results from the analyses of OR168::Tn*5* exconjugants revealed the presence of RP4-derived *mob* fragment. The results from these three different studies emphasises the need to screen presumptive exconjugants for the presence of donor vector DNA sequences when using pSUP-series vectors as the Tn*5*-delivery vehicle, especially in the mutagenesis of an unknown microorganism for the first time. In each case, the mechanisms involved seem peculiar to the bacterial strain and are probably the result of intrinsic vector activity and host recombination machinery acting in concert.

After the observations of Donald *et al.* (1985), one possible explanation of the presence of RP4-derived *mob* fragment in the exconjugants is that it is due to IS*50*-mediated cointegrate formation. In this study no OR168::Tn*5* exconjugant showed any DNA sequence homology to the p15A vector derivative, pACYC184. The result was as expected, and demonstrates the narrow host-range of the p15A-based vector, with pSUP1011 not being stably maintained in OR168::Tn*5* exconjugants. The result also demonstrates that total integration of the vector (cointegrate formation) did not occur, neither has inverse transposition (transposition mediated by IEs of Tn*5*). If either event had occurred, it would have resulted in the insertion of pACYC184-derived DNA in the exconjugants and would have been detected using the DIG-labelled pACYC184 vector as probe.

The presence of only the RP4-derived *mob* fragment, along with Tn*5* from the donor vector, infers that if cointegration had occurred, in every event a secondary precise deletion also occurred, resulting in the removal of all the pACYC184 sequence. The probability of 100% accuracy for this proposed secondary event seems remarkable. Therefore, it appears unlikely that the presence of the RP4-derived *mob* fragment is the result of vector cointegration. Unfortunately this cannot be clarified from the probing carried out in Section 3.8 because the 1.9-kb RP4-*mob* probe has only hybridized to a single restriction fragment. Although this has located the Tn*5* and the RP4-*mob* within the same restriction fragment, the location, if any, of the other 700 bp of the 2.6-kb RP4-derived *mob* fragment is also needed to ascertain precisely the origin of the DNA bordering both ends of the transposon within the exconjugants. The diagram of pSUP1011 shown in Figure 3.8.2 shows the transposon bordered at

both OE's by the 2.6-kb RP4-derived *mob* fragment. With cointegrate formation, at least one OE sequence of the transposon is juxtaposed to DNA of the target replicon. It is unknown for any of the three OR168::Tn*5* exconjugants analyzed, whether the Tn*5* element is bounded at only one or both OE's by donor DNA sequences. But the fact that the Tn*5* is bordered by donor sequences at one OE points to the acquisition of Tn*5* by these exconjugants by a mechanism other than conservative transposition, since conservative transposition results in target sequences directly bordering the transposon. It is also unclear whether cointegrate formation could be involved, since no exconjugant had more than one copy of the transposon. Cointegrate formation requires the existence of at least two copies of Tn*5* to allow for the chance of homologous recombination.

There was also a desire to probe the gel shown in Figure 3.8.1a with an internal DIG-labelled *SalI*/*Hin*dIII RP4-*mob* fragment from pSUP101, but because of loss of the *E. coli* clone containing the pSUP101 plasmid, it could not be done. The pSUP101 is the Tn*5*$^{-}$ parent vector from which pSUP1011 was derived (Simon *et al.*, 1983a). From this result, the extent of the RP4-*mob* fragment in the exconjugants could be determined unambiguously. It would also clearly show which sequences are bordering the inserted transposon, that is, whether it is donor or target DNA.

Another report of anomalous transposition activity by pSUP-series vectors was made by Schilf & Krishnapillai (1985). They reported the failure to obtain Tn*5* transposition in *Pseudomonas aeruginosa* using pSUP1011. However, mutagenesis was achieved via the pSUP1011 system provided there was complementation of RP4-*mob*::Tn*5* with an entire RP4-*mob* region. It was postulated from their observations that the initial failure to obtain Tn*5* mutants was due to the requirement of some feature encoded by the *mob* sequence that is essential for effective transposition in *P. aeruginosa*, but that was not functionally carried on pSUP1011. This raises the further question of whether they were observing, in the end, conventional transposition or an alternative anomalous event. By extension, does this suggest that the presence of the RP4-derived *mob* fragment in the OR168::Tn*5* exconjugants is due to the requirement of some function encoded by that RP4-derived *mob* fragment?

However, a more recent study by Goldberg *et al.* (1990) reported the general instability of Tn*5* in *P. aeruginosa* and noted its frequent and precise excision from the genome of this organism. This suggests the activity of a host-specific endonuclease acting specifically on long terminal inverted repeat sequences. This perhaps partly explains the observations made by Schilf & Krishnapillai (1985). It appears that the outcome of Tn*5* transposition events is influenced by the different recombination and DNA repair mechanisms of the host cell. Goldberg *et al.*

suggested *recBC*-analogous gene products may be responsible for Tn*5* instability in *P. aeruginosa* (Lundblad *et al.*, 1984).

But an explanation is still required for the presence of the 2.6-kb RP4-derived fragment from pSUP1011 in many of the OR168::Tn*5* exconjugants. It appears from the restriction mapping of the RP4-*mob* insertion site in three exconjugants that the insertion site is the same in each case, as the data in Table 3.8.1 show the same sized fragments hybridising to pSUP1011-specific probes. Because of the variable sizes of the long *Eco*RI fragments for the exconjugants, the existence of an insertional 'hot spot' for the RP4-*mob*::Tn*5* on the pOR168d plasmid cannot be ruled out, but requires an *ad hoc* hypothesis that rearrangements occur in the neighbourhood of the Tn*5* insertion. With no evidence of sequence homology between pSUP1011 and OR168, there is the suggestiuon that the insertion mechanism is not simply a reciprocal cross-over event.

4.1.4 Suggestions for the Mechanism Causing the Observed Plasmid Rearrangements

There is some debate over the actual mechanism for Tn*5* transposition. Much of the experimental evidence to date points to Tn*5* transposing via a conservative mechanism (reviewed by Berg, 1989). There has been some presumptive evidence, based primarily on observations of cointegrate formation, to support the notion that Tn*5* can also undergo replicative transposition (Ahmed, 1986; Isberg & Syvanen, 1981; Hirschel *et al.*, 1982). But these observations have been dismissed as products of the concerted events of homologous recombination and conservative transposition. Homologous recombination results in the formation of dimeric plasmids. This is followed by conservative transposition of just a segment of the dimer (Berg, 1983). Other support for a replicative mechanism for Tn*5* is founded on the presence of adjacent deletions, which are thought by some to arise only through the formation of a cointegrate intermediate. Proponents supporting a conservative mechanism suggest adjacent deletions are the product of aborted transposition events (Jilk *et al.*, 1993; Tomcsanyi *et al.*, 1990).

But a simple conservative mechanism cannot be used satisfactorily to explain the observations made with Tn*5* insertion into the pOR168d plasmid. Classical conservative transposition results in one copy of the transposon located in the target replicon with no other sequences from the donor molecule. So can a replicative mechanism explain the observations? As shown in Figure 1.5.2, replicative transposition begins with an initial 9 bp staggered cleavage of the target DNA and precise single-stranded nicks at the 3′-OE's of the transposon. After strand exchange

and ligation between the 5′-phosphate groups of the target and the 3′-hydroxyl groups of the transposon, a 'Shapiro intermediate' structure is formed. Cointegrate formation follows semi-conservative replication of the transposon, which results in the two parental replicons bounded by direct repeats of the transposon. This is the classical model for replicative transposition proposed by Shapiro (1979). No cointegrates with this structure were observed in the OR168::Tn*5* exconjugants screened, suggesting that if formed, they are unstable. However, the most appealing aspect about pursuing a replicative mechanism is that cointegrate formation is an essential intermediate step and places the transposon, the target DNA, and the RP4-*mob* fragments all within the same molecule.

The structures depicted by the restriction mapping of exconjugants shown in Figure 3.8.2 can be partially explained by using the model for replicative transposition as a starting point. The resolution of the cointegrate structure would be dependent on the replication machinery of the host. In the usual course of replicative transposition, resolution is expected to produce the donor plasmid, pSUP1011, and the target replicon containing a single copy of the transposon (as is shown by Tn*5* insertion into DH120 and DH225). Because pSUP1011 cannot replicate in the OR168 host, it would be lost from the population. The inclusion of the RP4-*mob* fragment in the target plasmid has to be caused by excision or exonuclease degradation of pACYC184 sequence following cointegrate formation and prior to, or as a consequence of, cointegrate resolution. This may be initiated by a secondary synapsis at the *oriT* site within the RP4-*mob* fragment, followed by excision of pAYC184 sequence and religation within *mob* fragment. This event then has to be followed by precise excision of one copy of the transposon. The end product is a target plasmid containing Tn*5* which is bounded by 9-bp direct repeats, with one of the repeats juxtaposed to the RP4-*mob* fragment.

An alternative mechanism resulting in the integration of the RP4-*mob* fragment into the target replicon may involve a site within the *mob* sequence acting as a surrogate Tn*5* end for a one-ended transposition event. This may reflect a preference for the *cis*-acting transposase to recognise this site instead of the usual outer ends of the Tn*5*. This would result in the fusion of both target and donor replicons without the duplication of the transposon. For this hypothesis to accurately represent the observed exconjugant products, replicon fusion has to be followed by recombination events to precisely remove the pACYC184 donor sequence.

An alternative model for cointegrate formation has been proposed that involves conservative transposition from the theta intermediate produced during rolling circle replication of some plasmids (Lichens-Park & Syvanen, 1988). This mechanism

seems unlikely in the case of pSUP1011, because this plasmid is unable to replicate in the OR168 host.

4.2 Areas Requiring Further Investigation

No clear conclusion can be reached for the mechanisms responsible for the anomalous insertion of the RP4::Tn*5* sequences in plasmids pOR168c and pOR168d of the exconjugants. None of the reported events of anomalous Tn*5* transposition correspond to the observations characterised in this study of OR168::Tn*5* exconjugants. To determine whether the acquisition of Tn*5* by the OR168::Tn*5* exconjugants is due to a replicative or a conservative mechanism for transposition, or indeed, some completely different mechanism, more work is needed. Clarification of this point will also presumably help determine the mechanism of acquisition of the RP4-*mob* fragment as well.

Future work to clarify some of the perplexing observations made in this study must include DNA sequencing of the region bordering the insertion sites, within both the OR168::Tn*5* exconjugants, and compare the sequences to both the OR168 parent and pSUP1011 sequences. Sequencing of the insertion site should reveal the presence of 9-bp direct repeats of target DNA at either end of Tn*5* if a transposase-mediated transposition event is responsible for the acquisition of Tn*5*. Accurate sequencing of the Tn*5* insertion sites can be easily achieved using the method developed by Ahmed (1987). But this would necessitate the recovery of the Tn*5*-bearing plasmids from the exconjugants, as well as plasmid pOR168d from the parent. This will necessitate optimising the plasmid isolation and recovery protocols of Hayman & Farrand (1990) for OR168 and its exconjugants.

Alternatively, the sequencing of the region bordering the insertion site could be achieved by conducting a more detailed restriction enzyme study of the insertion site, followed by subcloning appropriate fragments into sequencing vectors. Once the appropriate fragments have been obtained, they can be used as probe sequences to recover the homologous region from the OR168 parent. This sequencing is also required to provide the comparative means for identifying alterations at the molecular level, which may have contributed to the plasmid rearrangements.

The apparent sequence duplication in plasmids pOR168c and pOR168d, as evident from the sizes of the restriction fragments hybridising to the Tn*5* probe DNA in Table 3.7.2, also needs closer examination. This may also help explain the

insertional 'hot spot' for the RP4-*mob*::Tn*5* fragment. The presence and location of duplicated sequences on different replicons may identify a cause for the observed plasmid rearrangements. This investigation will require obtaining the conjectured duplicated segment and using it to probe both Eckhardt and genomic gels to determine the extent of the sequence duplication.

It has to be remembered that because of limitations in the selective procedure, it is implicit that not all manifestations and products of the recombination process will be isolated. The variations in plasmid profile observed in this study are difficult to explain within the bounds of current concepts of transposition and recombination. For instance, only Tn*5*-bearing exconjugants from the heterogeneric crosses were selected by using a specific antibiotic resistance marker. Isolation of OR168 exconjugants, other than Tn*5*-bearing ones, may be achieved by using spectinomycin in the media when selecting for recipients of the pSUP1011 plasmid, and then screening by colony hybridisation using, for example, the RP4-*mob* fragment as a probe.

As a corollary, it would also be interesting to compare, in detail, the structures and functions of the 58 kDa Tn*5* transposase and the 11 kDa TraI gene product of RP4. Both proteins are required for the precise single-stranded synapsis of DNA: Tnp at OE/IE sites of IS*50*, the TraI gene product at the *oriT* site within the *mob* sequence. Because both proteins have similar functions, there may be fundamental similarities between their structures and target sites. Transposase activity is critical for transposition. The protein is involved in binding to end sequences of the transposon which it brings together by a process of protein oligomerisation. It also utilised in cutting or nicking the DNA adjacent to the end sequences and inserting the transposon into the target DNA site. The 11 kDa TraI protein is a dimeric DNA binding protein and may function to direct specific interactions of other TRA1 region proteins (TraJ andTraH) in the formation of the relaxosome.

The obvious plasticity of the derived plasmid 'd', that has resulted in the 'laddering' observed in Figures 3.3.1 and 3.3.2, also begs closer examination in the general context of plasmid population dynamics. By using other well defined plasmids, such as RP4 and pBR322, for which probe sequences are readily available, the extent of this phenomenon can be assessed. The observed 'laddering' may be a normal conformational polymorphism or length (concatemer) polymorphism.

4.3 Conclusions

The principle result evident from this study is that the soil isolate, OR168, can acquire Tn*5* from the suicide vector pSUP1011 by means other than classical conservative transposition. With our current understanding and knowledge of Tn*5* transposition no simple model can be proposed to explain all the observations made in this study. The result does not dispute the conservative mechanism for Tn*5* transposition proposed by Berg (1989). In fact, all the transposition events isolated involving a chromosomal target, as well as two involving a plasmid target, do fit the conservative model. The observations accentuate the essential role of host-mediated recombination factors for the acquisition of Tn*5* from a specific vector.

Associated with the acquisition of the Tn*5*, by either the classical or anomalous mechanism, is substantial plasmid rearrangement. No definitive explanations can be made regarding the anomalous transposition events seen after the conjugative transfer of the pSUP1011 vector into OR168 without further investigation along the lines proposed within the general discussion.

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