

Molecular Studies on the TOL Plasmid of  
Pseudomonas putida (arvilla) mt-2

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## ABSTRACT

The TOL plasmid pWWO from Pseudomonas putida (arvilla) mt-2 exhibits several structural features, three of which form the basis of this thesis. The specificity with which a 40 kb segment of TOL DNA is lost from pWWO to yield the apparently cryptic plasmid pWWO-8 is shown here to be due to reciprocal recombination between a pair of directly repeated sequences positioned at the ends of the region to be excised. Heteroduplex analysis reveals that this repeated sequence is 1.4 kb in size, one copy of which is present in pWWO-8 at the point where the excision event has occurred.

After the isolation, in vivo, of RP4-TOL hybrid plasmids, several investigators alluded to the existence of a 'TOL transposon'. The most likely candidate for such an element was the 40 kb excised segment as this was thought to carry all the tol genes. It is demonstrated here that of six independently isolated RP4-TOL hybrid plasmids, all carry segments of TOL DNA larger than 40 kb which overlap both ends of the excised region. The mechanism of formation of these plasmids is discussed.

The observation that a plasmid free strain derived from PAW1 (which contains the TOL plasmid pWWO) showed homology to the TOL plasmid in DNA-DNA hybridisation studies prompted an analysis of three such independently isolated strains. Of these, one had a chromosomally located segment of 16 kb comprising a contiguous piece of pWWO-8 DNA; a second contained two similar segments in different chromosomal locations; the third isolate exhibited no such homology to TOL DNA. PAW1 (the parental strain) itself appears to contain chromosomally integrated TOL DNA sequences with similar end points to those found in the plasmid free strains.



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### Abbreviations

bp	base pair
C <sup>o</sup>	degrees centigrade
cpm	counts per minute
K	X1000
<u>tol</u>	indicates those genes encoding the toluene degradative function
Tol <sup>+</sup> (-)	indicates the ability of a strain to utilise meta-toluic acid as a sole source of carbon
TOL	is the name given to the plasmid specifying the above function
v	volume
w	weight



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### 1.1 Plasmids and Degradation

#### The genus *Pseudomonas* and biodegradation

Environmental pollution by a large number of recalcitrant compounds is a problem which has increased in severity during the last decades. This is due, in the main, to the dissemination by man of large quantities of organic chemicals in the form of by-products of industrial processes. These industrial effluents, as well as herbicides, pesticides and dye-stuffs, can accumulate in food chains and may be toxic to living organisms. There is therefore growing public concern regarding the control of levels of such chemicals in the environment.

At present these compounds, which constitute a potentially useful resource, have to be destroyed by expensive, high-energy processes in order that non-toxic products are released. Other ways of tackling this problem are now being investigated based on the ability of various microorganisms to degrade complex organic compounds. The soil bacteria play a major role in biodegradation and the members of the genus *Pseudomonas* are one of the most important groups in this regard and have long since been recognised as being among the most nutritionally versatile bacteria (Stanier *et al*, 1966). For example, over one hundred organic compounds (of three hundred tested), not including simple sugars and amino-acids, can be used as sole carbon sources by the aerobic *Pseudomonads* such as *P.aeruginosa*, *P.putida* and *P.fluorescens* (Stanier *et al*, 1966).

The biochemistry of the degradation functions exhibited by these organisms has been well documented (Clarke and Richmond, 1975), and



manipulation of genes encoding these degradative pathways is now possible due, mainly, to the following developments.

1. As several degradative pathways were found to be encoded by very large plasmids (for review see Chakrabarty, 1976), plasmid isolation techniques were developed enabling the DNA encoding these functions to be isolated for physical characterisation (Duggleby et al, 1977; Hansen and Olsen, 1978).
2. Powerful methods, such as gene cloning and transposition mutagenesis, have been developed for the analysis of gene structure and function (Kleckner et al, 1977; Bagdasarian and Timmis, 1981).

There is both scientific and industrial interest in degradative plasmids because of their role in the evolution of bacterial metabolism and their potential use in genetic engineering to produce organisms for pollution control and chemical syntheses. Evolution of degradative pathways in bacteria is enhanced by various genetic rearrangements which take place between degradative plasmids (Chakrabarty and Friello, 1974; Chakrabarty et al, 1978; Thacker and Gunsalus, 1979) and it has been shown that some of these rearrangements resemble transposition (Chakrabarty et al, 1978; Jacoby et al, 1978). Transposable elements have been shown to mediate a variety of different types of DNA rearrangements (Kleckner et al, 1977) and it has been proposed that such rearrangements contribute significantly to the reorganisation of bacterial genomes (Kleckner and Ross, 1980).

The TOL plasmid (pWWO) of Pseudomonas putida (arvilla) mt-2 exhibits several properties which make it a suitable subject for the analysis of DNA rearrangements occurring in degradative plasmids. These are:



1. Specific excision of a 40 kb DNA segment of the TOL plasmid (Bayley et al 1977).
2. The interaction between TOL and the various resistance plasmids including RP4, R91 and R2 (White and Dunn, 1977; Chakrabarty et al, 1978; Jacoby et al, 1978; Nakazawa et al, 1978; Jeenes and Williams, 1981).
3. The integration of a segment of TOL DNA into the chromosome of P.putida strain PAW1 (this study).

The molecular analysis of these three structural rearrangements is the subject of this thesis. I have therefore limited the scope of the introduction to a description of the degradative plasmids in Pseudomonas and of some structural properties of plasmids in general, which directly relate to the phenomena exhibited by the TOL plasmid.

#### Degradative plasmids

The degradative plasmids of Pseudomonas encode enzymes involved in the biodegradation of a wide range of organic compounds (Chakrabarty, 1976). The first degradative function demonstrated to be plasmid-encoded was salicylate degradation. This function could be lost spontaneously from cells and could be transferred to other strains of Pseudomonas (Chakrabarty, 1972). The discovery of this SAL plasmid was followed by that of plasmids called OCT (Chakrabarty, 1973), NAH (Dunn and Gunsalus, 1973), CAM (Rheinwald et al, 1973) and TOL (Williams and Murray, 1974; Wong and Dunn, 1974), encoding the degradation of alkanes, naphthalene, camphor and toluene respectively.

All these degradative plasmids can be transferred by conjugation to other strains of P.putida and so it has been possible to establish



whether these plasmids can stably co-exist in the same cell. In this way they have been assigned to incompatibility groups; whereas NAH, SAL and TOL belong to IncP-9 (Jacoby, 1977), CAM and OCT are members of the IncP-2 group (Jacoby, 1977). The camphor and alkane degrading functions of the CAM and OCT plasmids can be stably maintained in a single cell due to the fusion of these two replicons (Chakrabarty, 1973). This had led to the construction of a strain, containing both the CAM-OCT fused plasmid and an IncP-9 group plasmid, which has the capability to grow on a large variety of substrates found in crude oil (Friello et al, 1976).

The enzymes encoded on the CAM plasmid convert camphor to isobutyrate which, in turn, is degraded by chromosomally-encoded enzymes induced by this substrate (Rheinwald<sup>et al</sup>, 1973). It remains unclear whether there is some isobutyrate degrading function encoded on the plasmid, as CAM does encode the degradation of camphor past isobutyrate in some strains (Chakrabarty and Gunsalus, 1971). It has been proposed that whereas the structural genes reside on the plasmid, the regulatory genes are chromosomally located (Rheinwald et al, 1973).

The OCT plasmid also encodes an incomplete inducible pathway degrading alkanes to aldehydes which are then broken down to fatty acids by chromosomally-encoded enzymes (Grund et al, 1975; Nieder and Shapiro, 1975). P.putida strains carrying the OCT plasmid can grow on n-alkanes of 6 to 10 carbon atoms, these compounds acting as inducers for the plasmid-encoded function.

Unlike CAM and OCT, the NAH, SAL and TOL plasmids all encode complete degradative pathways. In each case the pathway includes the cleavage of an aromatic ring in the meta position, a rare mode



of ring fission (Stanier et al, 1966). The NAH and SAL plasmids have been shown to be closely related by DNA homology studies (Heinaru et al, 1978), and as salicylate is an intermediate in naphthalene degradation, it is interesting to speculate that one was derived from the other by a single addition or deletion of genetic material. Preliminary evidence however (Lehrbach and Broda, personal communication) would rule out this possibility.

#### The degradation of toluene and xylenes

Plasmids encoding the degradation of toluene and xylenes are widely distributed in nature (Worsey and Williams, 1975). Duggleby et al (1977) demonstrated by restriction enzyme analysis that several different plasmid isolates were identical to the archetypal TOL plasmid pWWO, indicating that this plasmid is often present in distinguishable natural isolates of Pseudomonas putida.

The TOL plasmid pWWO, from Pseudomonas putida (arvilla) mt-2 has become a model for both the structural and functional analysis of degradative plasmids (Williams and Murray, 1974; Worsey and Williams, 1975; Bayley et al, 1977; Benson and Shapiro, 1978; Chakrabarty et al, 1978; Downing et al, 1979; Downing and Broda, 1979; Nakazawa et al, 1980; Inouye et al, 1981; Bagdasarian and Timmis, 1981; Jeenes and Williams, 1981; Meulien et al, 1981). This plasmid is a suitable subject for such investigations for several reasons. Firstly, the degradative pathway it encodes is an important one as it includes the channelling of a variety of aromatic substrates through catechol, the meta cleavage of the aromatic ring and the breakdown of ring cleavage products to common metabolic intermediates (Williams and Murray, 1974). Williams and



Murray (1974) showed that strains having lost the plasmid encoded functions, retain the ability to grow on benzoate since it can be degraded by a second route, the chromosomally encoded ortho cleavage pathway which was known to exist in many species of Pseudomonas (Ornston and Stanier, 1966). A summary of both pathways is shown in Fig. 1.1. In strains carrying the plasmid, benzoate is metabolised via the meta pathway since it serves as the inducer of catechol-2,3 oxygenase and the meta pathway enzymes (Williams and Murray, 1974). Induction of the ortho pathway requires the initial conversion of benzoate to catechol which must then be converted, by basal levels of catechol-1,2 oxygenase, to cis-cis muconic acid, the product inducer of the chromosomal pathway (Ornston, 1966). Thus the ability to obtain strains which have been cured of their TOL function after growth on benzoate is possible because the second pathway which promotes the dissimilation of benzoate, is more efficient than the plasmid-borne function. There is therefore a selective advantage for cells using the chromosomally-encoded pathway (Williams and Murray, 1974).

Interactions between degradative pathways from different species have been investigated by introducing degradative plasmids into new hosts. It is hoped that by this means, new nutritional capabilities may be acquired by the host strains. Reinicke and Knackmuss (1980), have described derivatives of Pseudomonas sp. B13, a 3-chloro-benzoate degrading, strain which had acquired the capability to utilise 4-chloro and 3,5-dichlorobenzoate, notoriously xenobiotic compounds, <sup>this nutritional versatility arose</sup> as a consequence of introducing the TOL plasmid into this strain. The degradation of these substrates was shown to depend upon the combined activities of enzymes from both donor and recipient



Fig. 1.1 Summary of both chromosomal and plasmid-encoded aromatic ring-cleavage pathways operating in Pseudomonas spp. outlining their differences.



Fig 1.1

Plasmid encoded

Chromosomally

encoded

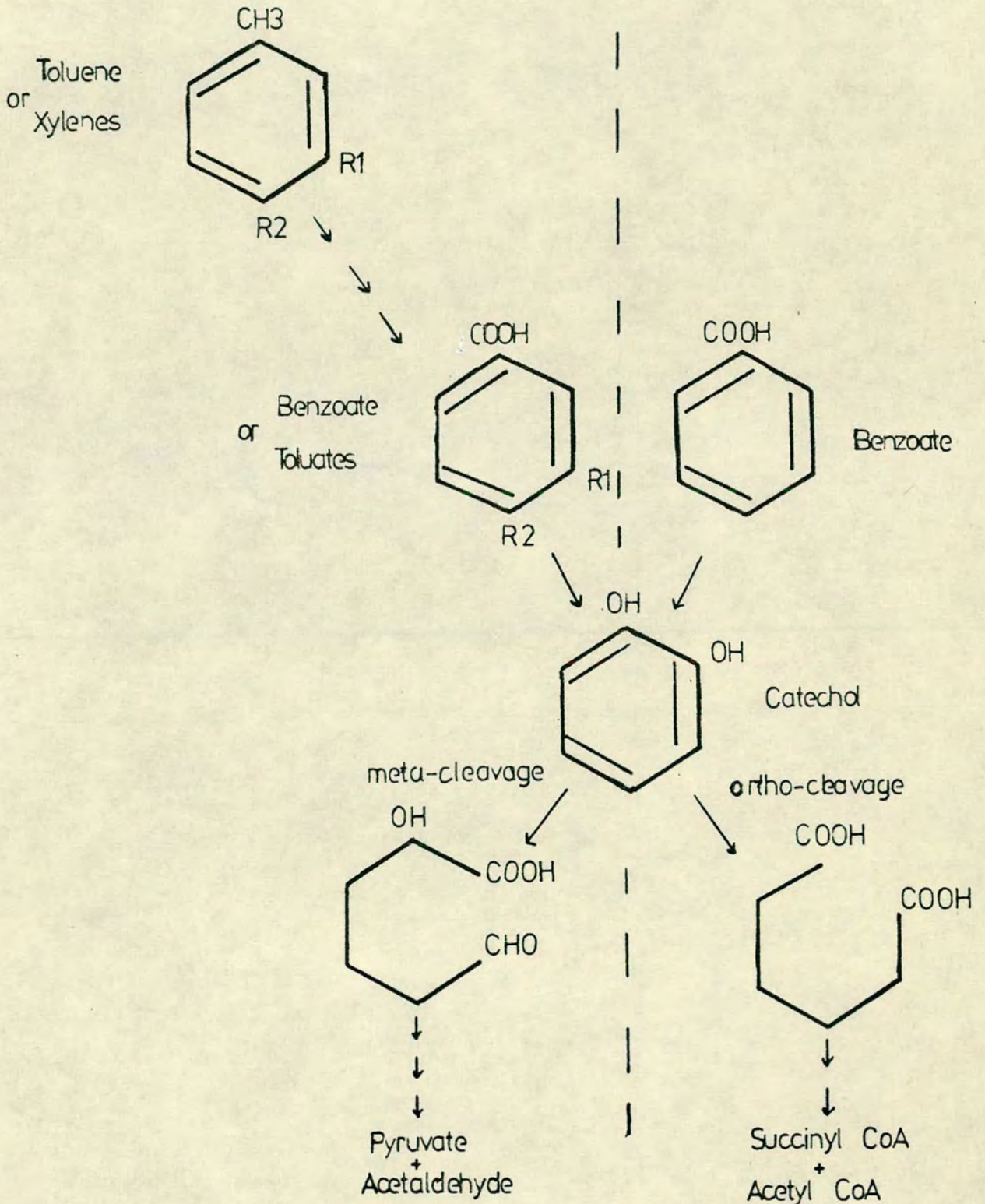
Features

wide substrate range

slow growing

narrow substrate range

fast growing





bacteria. Whereas the toluate deoxygenase of the TOL plasmid was induced, the remainder of the meta cleavage enzymes had become lost due to a block from the catechol2,3-deoxygenase downwards. This block is essential in order that the cell may survive, as toxic intermediates are formed from chlorinated aromatic substrates if the meta pathway is functional. This fact is supported by findings that exposure to halogenated substrates seems to select for cells defective in the meta cleavage pathway (Wigmore and Ribbons, 1981).

Recently the existence of a plasmid encoding the complete degradation of 3-chlorobenzoate has been demonstrated (Chatterjee *et al*, 1981) which proceeds by a modified ortho pathway. As the status of the genes encoding 3-chlorobenzoate degradation in Pseudomonas sp. B13 is unclear, interesting questions are raised regarding the evolutionary relationships of the degradative genes present in these bacteria.

#### The functional analysis of the TOL plasmid

To investigate the possibility of using some or all of the genes encoded on a degradative plasmid to confer some useful function on different host bacteria, it is important to establish whether these genes from Pseudomonas are expressed in other genera. To carry out these studies, Benson and Shapiro (1978) constructed transposon insertion derivatives of the TOL plasmid using Tn401, which encodes ampicillin resistance to study the host range of the TOL plasmid and the expression of degradative genes in E.coli. While the TOL::Tn401 plasmid could be transferred to E.coli selecting for the transfer of ampicillin resistance, the exconjugants could not grow on m-toluate. Several reasons were suggested to explain this



observation. (i) Lack of sufficient expression of the degradative genes; (ii) the accumulation of substances toxic to E.coli; (iii) the instability of the plasmid in a new host. The latter two explanations were discounted as, firstly, E.coli cells harbouring TOL::Tn401 could grow on 0.2% m-toluate if glucose was provided as an alternative carbon source (Benson and Shapiro, 1978), and, secondly, TOL::Tn401 could transfer back to Pseudomonas putida resulting in the full expression of the degradative genes when grown on m-toluate, indicating that no irreversible alteration to the plasmid had occurred (Benson and Shapiro, 1978). Nakazawa et al (1978) subsequently showed that the tol genes present on an RP4-TOL hybrid plasmid were expressed at very low levels in E.coli. The conclusion from both studies indicated that there was some block in expression of degradative genes of the TOL plasmid in E.coli. A possible explanation for this would be differences in cell envelope structure as this is known to play a role in other degradative functions, such as the alkane system (Fennewald et al, 1978) involving the OCT plasmid.

The availability of cleavage maps of the TOL plasmid pWWO for the restriction endonucleases HindIII and XhoI (Downing and Broda, 1979) and for EcoRI (Lehrbach and Broda, unpublished) has allowed investigators to position several structural genes mapped by either analysing deletion mutants (Nakazawa et al, 1980) or using cloning techniques (Inouye et al, 1981; Franklin et al, 1981). The physical map of pWWO with the positions of the degradative genes is shown in Fig. 1.2. The distribution of these genes in two clusters and the enzymic analysis reported so far (Nakazawa et al, 1980; Inouye et al, 1981), supports the model for the regulation

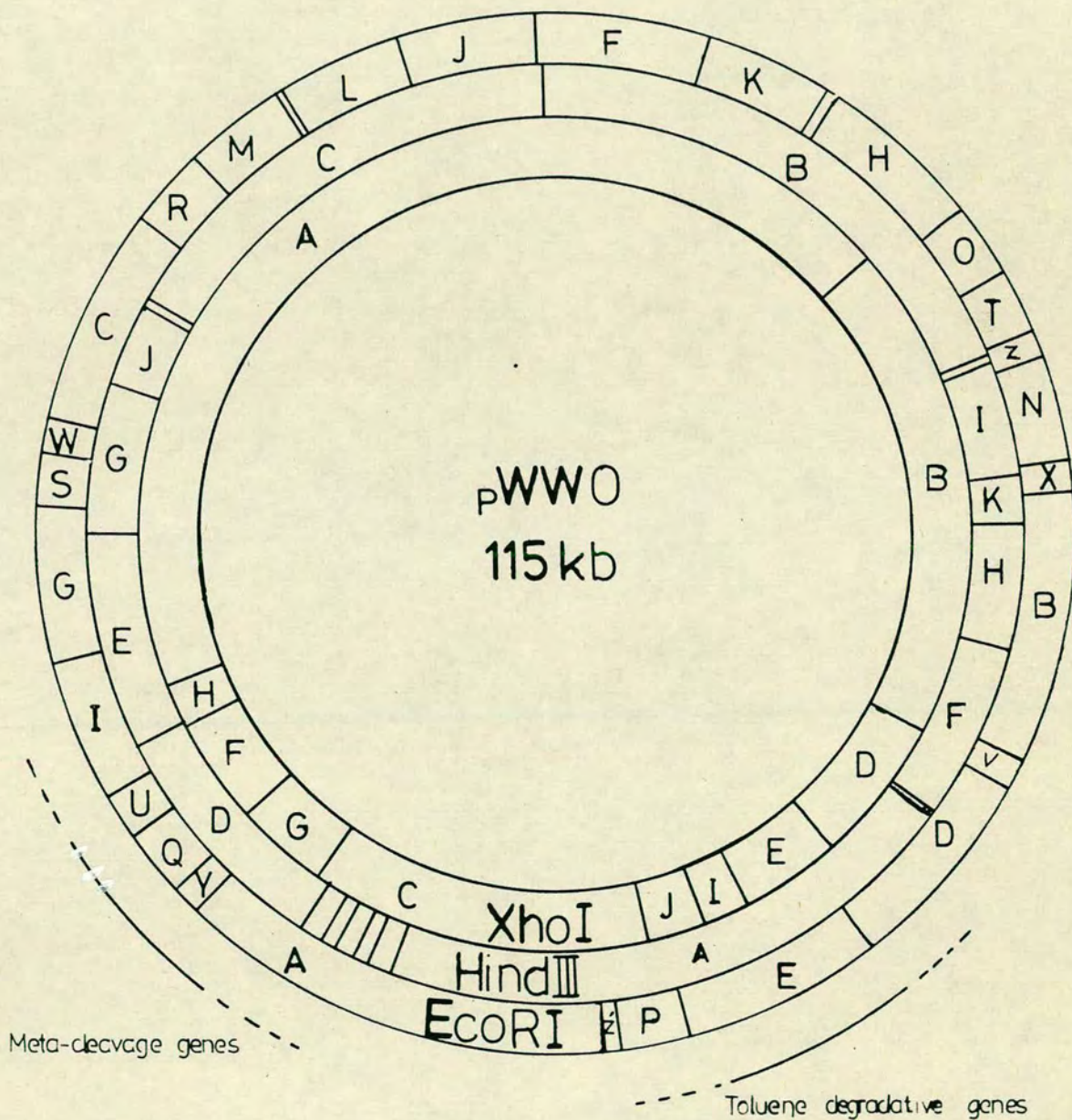


Fig. 1.2 Physical and genetic map of the TOL plasmid pWWO showing cleavage sites for the restriction endonucleases EcoRI, HindIII and XhoI, and the position of the tol genes. Fragments are lettered according to size (the smaller fragments are not labelled) based on their relative mobilities on agarose gels. The positions or sizes of the HindIII and XhoI generated fragments differ to those determined by Downing and Broda (1979) in that:

1. The estimated size of the plasmid has been reduced by 2 kb, to accommodate the EcoRI restriction mapping data, by altering the sizes of HindIII fragments HA and HC (HA = 23.5 kb, HC = 18.0 kb).
2. The positions of the XhoI fragments XE and XJ have been reversed as suggested by Nakazawa et al (1980) and confirmed by Jeenes and Williams (unpublished results).



Fig. 12





of the toluene degrading pathway proposed by Worsey et al (1978). That is: the enzymes are in two regulatory blocks xylABC and xylDEFG which are controlled by products of two regulatory genes xylR and xylS (see Fig. 1.3). The evidence for this was that when m-xylene was used as an inducer, all the meta pathway enzymes were fully induced whereas m-toluate induced only the enzymes responsible for its own degradation.

Inouye et al (1981) have cloned two structural genes xylB and xylE into the vector plasmid pBR322 and have found the enzymes encoded by these genes to be non-inducible in these hybrids, whereas the parental plasmid (an RP4-TOL hybrid plasmid) in E.coli exhibited the induction pattern characteristic of the wild type TOL plasmid. As the cloned fragments are themselves large (15.4 kb and 13.5 kb for xylB and xylE respectively), it would appear that the regulatory regions involved in the expression of these two genes are not located in the vicinity of the structural genes (Inouye et al 1981).

## 1.2 Plasmids and Genetic Rearrangements

Plasmids have been shown to exhibit several structural rearrangements including, (i) replicon fusions, as exemplified by the chromosome integration of the sex-factor F (Jacob and Wollman, 1958) or the fusion of RP4 and the Ti plasmids of Agrobacterium tumefaciens (Depicker et al, 1980; Hooykaas et al, 1980); and (ii) acting as recipients for transposons, e.g. in the transposition of the ampicillin resistance gene on TnA from RP4 (Hedges and Jacob, 1974).

These DNA-DNA interactions are promoted either by the host

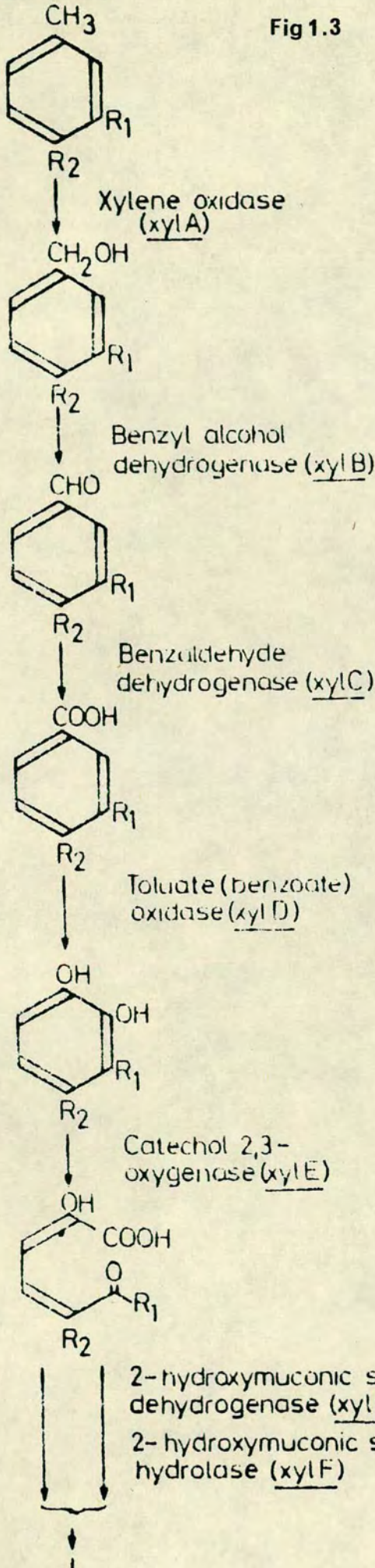


Fig. 1.3 The regulation of the toluene degrading pathway encoded by pWWO. This model (Worsey *et al.*, 1978) proposes that two regulatory genes xylR and xylS control two separate blocks of degradative genes as shown. Whereas inducers of the full pathway activate xylR, only the meta-cleavage enzymes are induced by substrates activating xylS.



Fig 1.3

PATHWAY



INDUCERS

Hydrocarbons  
Alcohols  
(Aldehydes)

GENES

xyl R

xyl A

xyl B

xyl C

xyl S

xyl D

xyl E

xyl F

xyl G

Carboxylic  
acids



recombination system, a prerequisite for which is the presence of some common DNA sequence in both replicons at which recombination can take place (Campbell, 1966), or by illegitimate recombination events, independent of the host function, the mediators of which are transposable elements (Starlinger and Saedler, 1976).

Transposable elements include three types of translocatable DNA segment (for review see Starlinger, 1980); (i) transposons, which carry a detectable genetic determinant, e.g. drug resistance (ii) bacteriophage Mu, and (iii) the insertion elements which are genetically recognised only when they insert into a structural gene or operon causing inactivation or polarity (Starlinger and Saedler, 1976).

Bacteriophage Mu has been termed a transposable element as it is able to integrate at random sites on the E.coli chromosome in the absence of host recombination systems (Faellen et al, 1971). This transposition of Mu is replicative and its replication does not require the excision of the Mu genome from its chromosomal site (Faellen et al, 1975).

The first transposons described were those carrying drug resistance genes, e.g. ampicillin on TnA (Hedges and Jacob, 1974; Bennett and Richmond, 1976), tetracycline on Tn10 (Kleckner, 1975), kanamycin on Tn5 (Berg et al, 1975), chloramphenicol on Tn9 (Gottesman and Rosner, 1975) and trimethoprim-streptomycin on Tn7 (Barth et al, 1976). Other determinants which have subsequently been shown to be located on transposons include resistance to mercuric ions on Tn501 (Stanisich et al, 1977) and enterotoxin production on Tn1681 (So et al, 1979). Transposition of these discrete units of DNA requires little or no homology between donor



and recipient replicons and, whereas in some cases the site of insertion of a transposon on the recipient inducer appears to be relatively non-specific (Kleckner et al, 1977), other instances show regional preference for transposon insertion (Grinsted et al, 1978).

The first reported insertion sequence, IS1, is 768 bp long (Ohtsubo and Ohtsubo, 1978) and was initially observed as the cause of a mutation in the gal operon in E.coli by Jordan et al (1968). All known insertion elements cause inactivation of structural genes or polarity in operons; however, IS2 carries a promoter sequence, and, if situated in the appropriate orientation, can activate adjacent genes (Saedler et al, 1974). The only characterised insertion sequence which has been associated with the genus Pseudomonas is IS21 (Willettts et al, 1981) found on the drug resistance plasmid R68.45 which was isolated from P.aeruginosa (Haas and Holloway, 1976).

#### The transposition process

The insertion sequences and transposons exhibit several common properties.

1. Precise excision of the element can occur as evidenced by the restoration of an impaired function (Starlinger and Saedler, 1976).
2. Inverted repeats in the DNA sequence appear at each end of these elements (Starlinger, 1980).
3. Transposition of these elements involves duplication of a short nucleotide sequence in the DNA at the target site of the recipient molecules (Grindley, 1978; Calos et al, 1978).
4. Transposition is a replicative process, the element remaining in its original location on the donor replicon (Bennett et al, 1977).



Several models for the mechanism of transposition have been proposed. These are based on studies of 'phage Mu replication and integration (Harshey and Bukhari, 1981), the analysis of the transposition of TnA (Arthur and Sherratt, 1979; Shapiro, 1979), or that of IS1 (Grindley and Sherratt, 1979). These models take into account the various DNA rearrangements which can be observed as a result of the activity of these elements (Starlinger and Saedler, 1976; Starlinger, 1980). The outlines of the Shapiro (1979) model are described here and shown diagrammatically in Fig. 1.4.

This model postulates, as the first step in the process, a single stranded cleavage at each end of the transposable element and also at each end of a short nucleotide sequence on the recipient replicon called the target. The two replicons are then fused and DNA synthesis ensues making a second copy of the element. At this stage each copy of the element is attached to a segment of donor molecule and a segment of recipient molecule. Reciprocal recombination occurs within the transposon, the donor molecule being regenerated and the element inserted at a new genetic site. This recombination does not require proteins needed for homologous recombination (RecA pathway) and in some instances, e.g. in TnA, it occurs by a transposon-encoded site specific recombination system (Arthur and Sherratt, 1979).

One significant difference between this model and that based on studies of replication intermediates of Mu 'phage after induction (Harshey and Bukhari, 1981), is that, in the Mu model only one end of the transposable element is attached to the target site, at which a staggered double-stranded cleavage has occurred. This allows



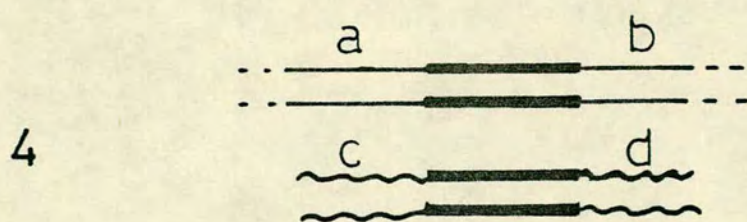
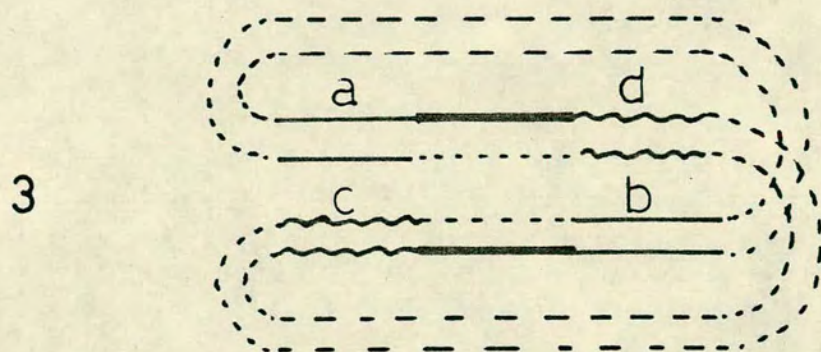
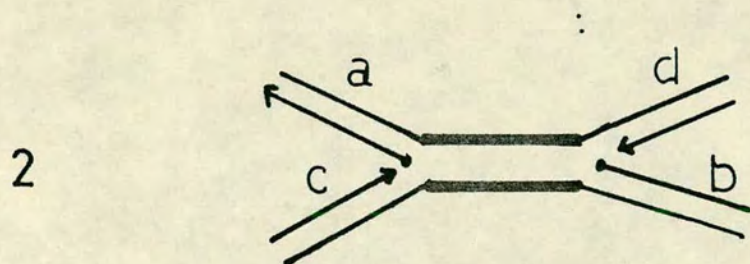
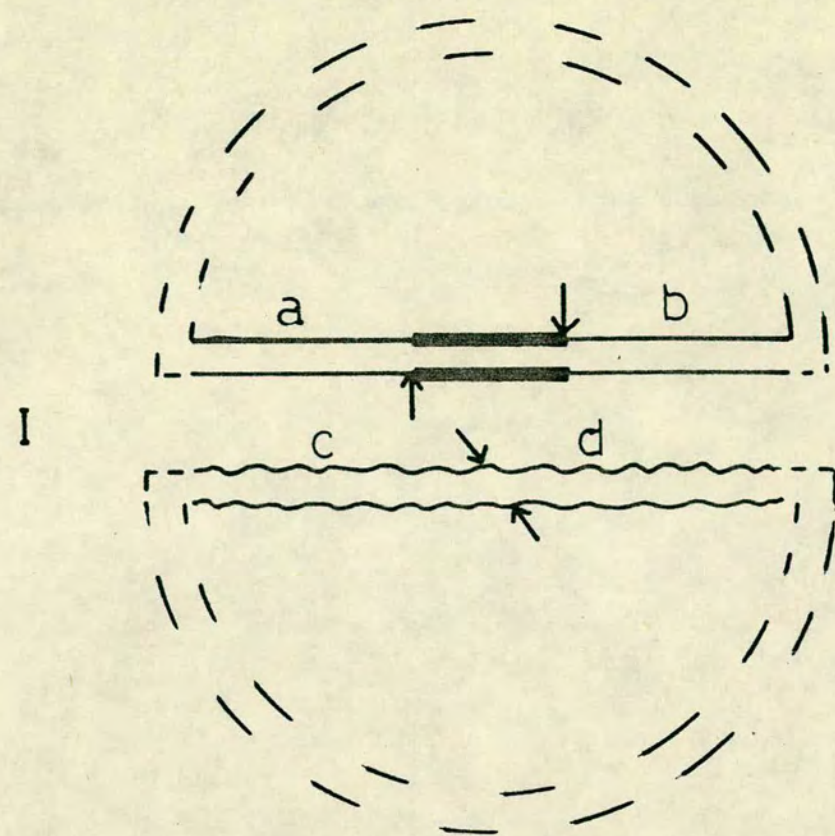
Fig. 1.4 A model for the transposition process (Shapiro, 1979).

The various stages envisaged are:

1. Single-stranded cuts are made at the ends of the transposable element, and a staggered cut made in the target DNA.
2. One end of the transposable element is attached by a single strand to each protruding end of the staggered cut. Two replication forks are thus created and replication may proceed to make a copy of the transposable element and duplicate a short nucleotide sequence at the target.
3. At this point, two copies of the transposable element are each attached to a segment of the donor molecule and a segment of the recipient molecule. If ab and cd were circles, abc and d would now be covalently connected and the transposable elements form the junctions of a fused replicon.
4. Reciprocal recombination between copies of the transposable element inserts the element at a new genetic site and regenerates the donor molecule. This recombination event is independent of the RecA pathway in E.coli.



Fig. I.4



← 3'-OH

← 5'-PO<sub>4</sub>



the transposable element to "roll" into place while replication of the element proceeds.

It is interesting to speculate to what degree transposition is independent of genes located outside transposable elements on the host chromosome. The small insertion sequences may be very different from the transposons (and larger IS's) in this respect as the latter have the capability to transpose in very unrelated hosts (Starlinger, 1980).

#### Genetic rearrangements mediated by transposable elements

The IS elements and transposons have been implicated in a variety of unusual recombination phenomena: deletions, inversions, replicon fusions, integrations of F factors and amplification of R factors (Starlinger and Saedler, 1976; Kleckner et al, 1977), and it seems likely that the ability of these elements to insert randomly into non-homologous DNA could play a role in genetic rearrangements in bacteria and indeed in higher organisms (Starlinger, 1980).

These DNA rearrangements can be explained by the models discussed above. Whereas replicon fusion results from the failure of the cointegrate, formed as an intermediate in the transposition process to resolve, deletions and inversions result from transpositions that are terminated by recombination, when the element transposes from one site to another in the same replicon. Deletions formed in this way have one end-point at the terminus of a transposable element, the other end-point being found at a variable distance from the element depending on the target site at which the transposition occurs (Reiford and Saedler, 1975).



The sex factor F mobilises the chromosome of E.coli by integrating into the chromosome (Jacob and Wollman, 1958) by means of recombination between homologous sequences present on both replicons (Campbell, 1966) forming Hfr strains. There are three discrete DNA sequences on F which can serve as attachment sites for such events. These are sequences homologous to IS2 and IS3 and  $\gamma\delta$  (Davidson et al, 1975). The  $\gamma\delta$  sequence has been shown directly, to be transposable from F (Guyer, 1978). Although IS2 and IS3 transpose from the E.coli chromosome, they have not been demonstrated to be active as transposable elements on F (Kilbane and Mallamy, 1980).

The identification of these sequences on F, was obtained from examination of F' plasmids which are sometimes formed when F, returning to its autonomous mode of replication, carries with it some chromosomal DNA. Heteroduplex analysis of F' plasmid molecules showed that IS2, IS3 or the  $\gamma\delta$  sequence is always present at the junction between the F genome and the host chromosome (Davidson et al, 1975).

It is known that Hfr formation can occur in recombination deficient strains (Cullum and Broda, 1979) however 99% of chromosomal integration of F was shown to be recA dependent and so the insertion sequences on F seem, for the most part, to act as portable regions of homology in Hfr formation.

Another type of plasmid-chromosome interaction is exemplified by R100 which is a large self-transmissible drug resistance plasmid. The resistance determinants present on R100, except for the tetracycline resistance gene which resides on the transposon Tn10, (Kleckner, 1975), are clustered between two directly repeated copies



of IS1 which can promote the transposition of this segment of the plasmid (Meyer and Iida, 1979). R100 can integrate into the chromosome by recombination between IS1 sequences present on the plasmid and on the chromosome, in an analogous way to Hfr formation (Nishimura et al, 1973).

It has been shown that R100 can integrate into the E.coli chromosome by another, rather novel, mode (Chandler, 1979), which is explained here. The tetracycline resistance gene in Tn10 is flanked by inverted repeats in the DNA sequence of about 1.4 kb. The 1.4 kb repeated segment, which has in turn small inverted repeats at each end, can act as a transposable element in its own right and has been designated IS10 (Kleckner et al, 1975; Kleckner et al, 1979). The transposition of the right hand repeat seems to be more frequent than that of the left and this has been correlated with the production of a gene product encoded by one repeat but not the other (Foster et al, 1981) Tn10 usually transposes using the outermost ends of the IS10 elements to define the ends of the transposon. If however the inner ends of the IS10 sequences immediately adjacent to the tetracycline resistance gene are used, the transposon becomes the whole of the R100 plasmid (except for the tet gene) and this can transpose into the chromosome by this so called inverse transposition process (Chandler, 1979). From the resulting Hfr strain, many extra-chromosomal circles can be produced from the r-determinant region of R100 defined by the two copies of IS1 present in direct repeat. These 'r-det' plasmids were shown to be incapable of autonomous replication (Silver et al 1980).

The first plasmid-chromosomal interaction to be studied in



Pseudomonas was that of R68.45 (Haas and Holloway, 1976). R68.45 is a mutant plasmid derived from R68 which is identical to the RP1, RP4 and RK2 plasmids of incompatibility group IncP-1 (Burkhardt et al, 1979). R68.45 arose by selecting for high frequency transfer of chromosomal markers from P.aeruginosa (Haas and Holloway, 1976). The only physical difference between R68, which mobilises the P.aeruginosa chromosome very poorly, and R68.45, is that there is a duplication of a 2.1 kb DNA segment near the kanamycin gene on R68.45 (Leemans et al, 1980; Riess et al, 1980). Transposition of this 2.1 kb segment (IS21) from R68.45 has been demonstrated by Willetts et al (1981), whereas the same sequence, which is present as a single copy on R68, either does not transpose, or does so at very low frequencies (Willetts et al, 1981).

IS21 gives R68.45 its chromosomal mobilising ability (Cma) and can be found in 'R68.45 prime' plasmids, flanking the R68.45-chromosomal DNA junctions as a direct repeat (Leemans et al, 1980). IS21 does not appear to be present in the P.aeruginosa chromosome (Willetts et al, 1981) and it is proposed that the formation of a cointegrate intermediate during transposition of IS21 from R68.45 to the bacterial chromosome is responsible for the Cma exhibited by this plasmid (Willetts et al, 1981). This mechanism offers an explanation as to how R68.45 mobilises the P.aeruginosa chromosome from multiple origins (Haas and Holloway, 1976; Haas and Holloway, 1978).

### 1.3 Structural Features of Degradation Plasmids - Aims of this Study

Several structural rearrangements exhibited by degradative



plasmids have been documented. For example when the OCT plasmid is transferred from its natural host P.oleovorans to P.putida, it is thought to dissociate into three separate replicons called OCT (as this confers the degradative function), MER and factor K (Chakrabarty, 1974; Chakrabarty and Friello, 1974). The OCT plasmid in P.putida behaves as a non-transmissible plasmid which can be mobilised by factor K. Factor K acts as a sex factor in P.putida and mediates the transfer of chromosomal genes (Mydroie and Chakrabarty, 1976). MER is a self transmissible plasmid encoding resistance to mercuric ions (Chakrabarty and Friello, 1974). The mechanisms for these rearrangements are unknown due to the lack of structural characterisation of these plasmids.

In contrast to this, the TOL plasmid, pWWO, is well characterised physically (Downing et al, 1979; Downing and Broda, 1979) and is ideally suited as a subject for genetic rearrangements as it exhibits several interesting structural properties. Firstly, the TOL plasmid undergoes a specific excision event involving 40 kb of DNA which abolishes the toluene degrading function and can be selected for by growing cells harbouring the TOL plasmid on benzoate. The resultant plasmid is apparently cryptic and its archetype is called pWWO-8 (Bayley et al, 1977). In Chapter 3 the elucidation of the molecular mechanism by which this event occurs is described.

Secondly, the TOL plasmid interacts with various resistance plasmids forming hybrids which carry the functional toluene degrading determinants encoded on pWWO (White and Dunn, 1977; Chakrabarty et al, 1978; Jacoby et al, 1978; Nakazawa et al, 1978; Franklin and Williams, 1980; Jeenes and Williams, 1981). In



Chapter 4 the molecular analysis of six independently isolated RP4-TOL plasmids are described and in Chapter 5 a model for the mechanisms of formation of these plasmids is presented.

Finally, after the chance observation that chromosomal DNA from certain plasmid-free strains of P.putida showed homology with the TOL plasmid in DNA-DNA hybridisation studies (D. Morris and P. Broda, personal communication), the question arose as to whether some plasmid-chromosome interaction was taking place. In Chapter 6, the status of the TOL DNA in these strains is investigated and the nature of the interaction discussed.



## CHAPTER 2 MATERIALS AND METHODS

2.1 Materials1. Bacterial strains and plasmids

A list of bacterial strains and plasmids used in this study is given in Table 2.1.

2. Media

L-broth contained 10 g tryptone (Difco), 5 g NaCl and 5 g yeast extract (Difco) per litre, adjusted to pH 7.2.

Minimal medium for growing Pseudomonas spp. was prepared by the addition of 1 M carbon source to 80 ml 4 x M9 salts to give a final concentration of 10 mM. This mixture was then added to 300 ml sterile distilled water to which had been added 0.5 ml stock salt solution containing 0.5 M  $\text{MgSO}_4$  and 18mM  $\text{FeSO}_4$ . For growing Escherichia coli on minimal medium 5 x Spitzizin salts were used, in place of M9, and the stock salt solution was omitted. Amino-acids were used at 20  $\mu\text{g/ml}$ , final concentration. For plates and slants, these media were solidified by the addition of 1.5% New Zealand Agar.

3. Buffers

Bacterial buffer: 3 g  $\text{KH}_2\text{PO}_4$ , 7 g  $\text{Na}_2\text{HPO}_4$ , 4 g NaCl and 0.2 g  $\text{MgSO}_4 \cdot 7 \text{H}_2\text{O}$  per litre.

TES: 50 mM Tris-HCl, 5 mM EDTA, 10 mM NaCl, pH 7.2

TE: 10 mM Tris-HCl, 1 mM EDTA, pH 7.2

TNE: 10 mM Tris-HCl, 10 mM NaCl, 2 mM EDTA, pH 8.0

SSC: 150 mM NaCl, 15 mM Na citrate

TBE (electrophoresis buffer): 89 mM Tris-HCl, 89 mM boric acid, 2.5 mM EDTA, pH 8.2.



Table 2.1      Bacterial Strains and Plasmids

<u>Bacterial strain</u>	<u>Relevant genotype</u>	<u>Plasmids and their characteristics</u>	<u>Size (kb)</u>	<u>Strain source/reference (a)</u> <u>Plasmid source/reference (b)</u>
<u>Pseudomonas putida</u>				
PAW1	prototroph	pWWO Tra <sup>+</sup> Tol <sup>+</sup>	115	Williams and Murray, 1974 (a,b)
PAW8	prototroph	pWWO-8 Tra <sup>+</sup> Tol <sup>-</sup>	75	Williams and Murray, 1974 (a) Bayley <u>et al</u> , 1977 (b)
PAW82	prototroph	-	-	Bayley <u>et al</u> , 1977
PAW85	prototroph	-	-	Bayley <u>et al</u> , 1977
PAW86	prototroph	-	-	Bayley <u>et al</u> , 1977
PAW153	<u>trp</u> <sup>-</sup>	pED3300 (RP4-TOL) Cb <sup>R</sup> Kn <sup>R</sup> Tet <sup>S</sup> Tra <sup>+</sup> Tol <sup>+</sup>	120	Franklin and Williams, 1980 (a,b)
PAW339	<u>trp</u> <sup>-</sup>	pED3301 (RP4-TOL) Cb <sup>R</sup> Kn <sup>R</sup> Tet <sup>S</sup> Tra <sup>+</sup> Tol <sup>+</sup>	120	Franklin and Williams (unpublished)
ED3305	<u>trp</u> <sup>-</sup>	pED3305 Cb <sup>R</sup> Kn <sup>R</sup> Tet <sup>S</sup> Tra <sup>+</sup> Tol <sup>-</sup>	80	This study
TN2002	prototroph	pTN2 (RP4-TOL) Cb <sup>R</sup> Kn <sup>R</sup> Tet <sup>R</sup> Tra <sup>+</sup> Tol <sup>+</sup>	110	Nakazawa <u>et al</u> , 1978 (a,b)
AC810	<u>met</u> <sup>-</sup>	pED3302 (RP4-TOL) Cb <sup>R</sup> Kn <sup>R</sup> Tet <sup>S</sup> Tra <sup>+</sup> Tol <sup>+</sup>	120	Chakrabarty <u>et al</u> , 1978 (a,b)
AC836	<u>trp</u> <sup>-</sup>	pED3303 (RP4-TOL) Cb <sup>R</sup> Kn <sup>R</sup> Tet <sup>R</sup> Tra <sup>-</sup> Tol <sup>+</sup>	115-120	A.M. Chakrabarty (unpublished)



AC34	<u>ade</u> <sup>-</sup>	-	-	A.M. Chakrabarty (unpublished)
A312	prototroph	-	-	Stanier <u>et al</u> , 1966
EDPS100	<u>str</u> <sup>-</sup>	-	-	C.J. Duggleby (unpublished)
NCIB10015	prototroph	-	-	Stanier <u>et al</u> , 1966
<u>Pseudomonas aeruginosa</u>				
PU21	<u>ilv</u> <sup>-</sup> <u>leu</u> <sup>-</sup> <u>str</u> <sup>-</sup>	pED3304 (RP4-TOL) Cb <sup>R</sup> Kn <sup>R</sup> Tet <sup>S</sup> Tra <sup>+</sup> Tol <sup>+</sup>	120	Jacoby <u>et al</u> , 1978 (a,b)
PA02	<u>ser</u> <sup>-</sup>	-	-	Holloway, 1969
PAC1	prototroph	-	-	Kelly and Clarke, 1960
<u>Escherichia coli</u>				
ED8654	<u>hsdR</u> <sup>-</sup> M <sup>+</sup> S <sup>+</sup> <u>met</u> <sup>-</sup>	-	-	Borck <u>et al</u> , 1976
JC411	<u>his</u> <sup>-</sup> <u>arg</u> <sup>-</sup> <u>leu</u> <sup>-</sup> <u>pyrB</u> B <sub>1</sub> <u>str</u>	-	-	Bachmann, 1972
ED2117	prototroph $\Delta$ lac <u>recA</u> F <sup>-</sup>	-	-	N. Willetts
ED2196	<u>his</u> <u>trp</u> <u>nal</u>	-	-	N. Willetts

Notes: Abbreviations: Amp - ampicillin    Cb - carbenicillin    Tet - tetracycline;    Tra<sup>+</sup> - transfer proficient  
Tol<sup>+</sup> - ability to utilise meta-toluate as sole carbon source.    Kn - Kanamycin



Table 2.1 (a)    E.coli strains carrying recombinant plasmids based on pBR322

<u>Strain</u>	<u>Plasmid</u>	<u>Cloned <u>Hind</u>III fragments of pWVO or pWVO-8*</u>
ED3306	pED3306	HD
ED3307	pED3307	HF
ED3308	pED3308	Hd*
ED3309	pED3309	HC
ED3310	pED3310	HE
ED3311	pED3311	HG
ED3312	pED3312	HI
ED3313	pED3313	HJ
ED3314	pED3314	HK

Notes: All strains are derivatives of ED8654 and are phenotypically Amp<sup>R</sup> Tet<sup>S</sup>.

These strains with the exception of ED3308 (this study) were obtained from

R.G. Downing and P. Broda (unpublished)



T<sub>4</sub> ligation buffer (x10): 60 mM Tris-HCl (pH 7.2), 10 mM EDTA,  
100mM MgCl<sub>2</sub>, 100 mM dithiothreitol (DTT), 10 mM ATP.

Nick-translation buffer (x10): 200 mM Tris-HCl (pH 7.4), 100 mM  
MgCl<sub>2</sub>, 600 mM NaCl.

Gel loading buffer: 10% (w/v) Glycerol, 0.025% (w/v) Bromophenol-  
blue, 0.05% (w/v) Xylene cyanol FF, made up in TBE buffer.

Restriction endonuclease reaction buffers are shown in Table 2.2.

#### 4. Enzymes

A list of restriction endonucleases used in this study is given  
in Table 2.3.

Bacterial Protease (Proteinase K) - Sigma Chemical Co Ltd.

Bovine pancreatic DNase I (crude grade) - Sigma Chemical Co Ltd.

E.coli DNA polymerase I - Mrs. Barbara Will.

Egg white lysozyme (grade I lyophilised) - Sigma Chemical Co Ltd.

Ribonuclease A (type I-A) - Sigma Chemical Co Ltd.

T<sub>4</sub> DNA ligase - Mrs. Sandra Bruce.

#### 5. Antibiotics

Ampicillin - Beechams Research Laboratories Ltd.

Carbenicillin (pyopen) - Beechams Research Laboratories Ltd.

Cycloserine - Sigma Chemical Co Ltd.

Kanamycin - Winthrop Laboratories Ltd.

Streptomycin - Glaxo Laboratories Ltd.

Tetracycline (achromycin) - Lederle Laboratories Division, Cyanamid  
of Great Britain Ltd.

Sulphonamide - Glaxo Laboratories Ltd.



Table 2.2    Restriction enzyme buffers

<u>Enzyme</u>	<u>Tris-HCl (pH 7.4)</u>	<u>NaCl</u>	<u>MgCl<sub>2</sub></u>	<u>KCl</u>	<u>β-Mercaptoethanol</u>
<u>AluI</u>	6	50	6	-	6
<u>BglII</u>	20	-	7	-	7
<u>EcoRI</u>	100	50	5	-	2
<u>HindIII</u>	20	60	7	-	-
<u>HpaI</u>	20	-	10	20	-
<u>KpnI</u>	6	6	6	-	6
<u>PstI</u>	20	-	10	-	-
<u>SalI</u>	8	150	10	-	-
<u>SmaI</u>	15	-	6	15	-
<u>XhoI</u>	8	150	6	-	6

N.B.    All concentrations in mM.



Table 2.3      Restriction Endonucleases

<u>Restriction Enzyme</u>	<u>Organism</u>	<u>Target sequence</u>	<u>Source</u>
<u>AluI</u>	<u>Arthrobacter luteus</u>	AG $\downarrow$ CT	A. Newman
<u>BglII</u>	<u>Bacillus globigii</u>	A $\downarrow$ GATCT	B.R.L.*
<u>EcoRI</u>	<u>Escherichia coli</u>	G $\downarrow$ AATTC	P. Ford
<u>HindIII</u>	<u>Haemophilus influenzae</u> Rd	A $\downarrow$ AGCTT	S. Bruce
<u>HpaI</u>	<u>Haemophilus parainfluenzae</u>	GT $\downarrow$ AAC	I. Garner
<u>KpnI</u>	<u>Klebsiella pneumoniae</u>	GGTAC $\downarrow$ C	B.R.L.
<u>PstI</u>	<u>Providencia stuartii</u>	CTGC $\downarrow$ A $\downarrow$ G	A.M.C. Brown
<u>SalI</u>	<u>Streptomyces albus</u> G	G $\downarrow$ TCGAC	This study
<u>SmaI</u>	<u>Serratia marcescens</u> sb	CCC $\downarrow$ GGG	Boehringer Mannheim
<u>XhoI</u>	<u>Xanthomonas holocola</u>	C $\downarrow$ TCGAG	B.R.L.

\* B.R.L.    Bethesda Research Laboratories



## 6. Reagents

Benzoic Acid - Aldrich Chemical Co Ltd.  
Caesium Chloride - Fisons Scientific Apparatus.  
Chloroform - Koch-Light Laboratories Ltd.  
Deoxyribonucleoside triphosphates - Boehringer, Mannheim,  
West Germany.  
EDTA - BDH Chemicals Ltd.  
Ethidium Bromide - Sigma Chemical Co Ltd.  
Formamide - Pharmacia.  
Isoamylalcohol - Koch-Light Laboratories Ltd.  
 $\beta$ -Mercaptoethanol - Koch-Light Laboratories Ltd.  
Phenol - Koch-Light Laboratories Ltd.  
Propan-2-ol - Koch-Light Laboratories Ltd.  
Sodium Chloride - Fisons Scientific Apparatus.  
Sodium Citrate - Fisons Scientific Apparatus.  
Sodium Dodecyl Sulphate - BDH Chemicals Ltd.  
Sodium Hydroxide (pellets) - Koch-Light Laboratories Ltd.  
D+ Sucrose - Koch-Light Laboratories Ltd.  
m-Toluic acid - Aldrich Chemical Co Ltd.  
Trizma-Base (Tris) - Sigma Chemical Co Ltd.  
Triton X-100 - Sigma Chemical Co Ltd.

## 7. Miscellaneous

Acrylamide - BDH Chemicals Ltd  
Agarose - Sigma Chemical Co Ltd.  
Chromatography paper Whatman 3mm - Whatman Ltd.  
Dowex 50W-X8, 20-50 US Mesh (17) - BDH Chemicals Ltd.  
Nitrocellulose filters (type BA85) - Schleicher and Schull,  
West Germany.



Radio Chemicals,  $\alpha$ - $^{32}\text{P}$  dNTP with activities of 1  $\mu\text{Ci}/\mu\text{l}$  in ethanolic solution were obtained from Amersham Radiochemicals Ltd. Repelcote - Hopkin and Williams.

## 2.2 Bacterial Techniques

A. Growth conditions. Escherichia coli was grown at 37°C with aeration in flasks containing L-broth or on nutrient or minimal agar plates. Antibiotics were used at the following concentrations: ampicillin or carbenicillin, 50  $\mu\text{g}/\text{ml}$ ; tetracycline, 20  $\mu\text{g}/\text{ml}$ ; kanamycin, 50  $\mu\text{g}/\text{ml}$ ; sulphonamide, 100  $\mu\text{g}/\text{ml}$ .

Pseudomonas aeruginosa was normally grown at 37°C with aeration as described for E.coli. Pseudomonas putida was grown at 30°C with aeration as described for E.coli. Antibiotics for Pseudomonas spp. were used at the following concentrations: ampicillin or carbenicillin, 2 mg/ml; tetracycline, 50  $\mu\text{g}/\text{ml}$ ; kanamycin, 50  $\mu\text{g}/\text{ml}$ ; streptomycin, 1 mg/ml.

B. Transfer of conjugative plasmids. In all mating experiments, exponentially grown broth cultures of donor and recipient cells were spotted together on a dry nutrient agar plate and grown overnight. They were then scraped off with a sterile spatula, diluted in bacterial buffer, and plated out on the appropriate selective plates.

## 2.3 DNA Techniques

A. Isolation of plasmid DNA. Three methods of plasmid DNA isolation were employed in this study for the following reasons. Method (i) is that of Birnboim and Doly (1979), and was used to



screen for plasmids of all sizes. Preliminary physical characterisation of plasmid DNA isolated in this manner is possible, as the method yields DNA of sufficient purity to undergo digestion with endonucleases. Method (ii) was performed when large quantities of pure plasmid DNA were required from strains carrying small plasmids of sizes in the range 4-20 kb. It is essentially the method of Guerry et al (1973). Method (iii) is a modification of that of Hansen and Olsen (1978), which was designed specifically for the isolation of large resistance and degradative plasmids, i.e. those over 50 kb in size.

Method (i). One ml of an early stationary phase culture of the plasmid containing strain was spun for 15 seconds in an Eppendorff microcentrifuge (12 K x G). The pellet was resuspended in 100  $\mu$ l of lysis solution containing 2 mg/ml lysozyme, 25 mM Tris-HCl (pH 8.0) 10 mM EDTA and 50 mM glucose. The resuspended cells were left standing on ice for 30 minutes. 200  $\mu$ l of a solution containing 0.2 M NaOH and 1% SDS was then added to lyse the cells, the mixture clearing and becoming viscous immediately. The lysate was left standing on ice for 5 minutes after which 150  $\mu$ l of high salt solution (3 M Na acetate, pH 4.8) was added and the contents of the tube were gently mixed until a heavy coarse precipitate formed. The mixture was left standing on ice for a further 60 minutes with occasional mixing and then centrifuged for 5 minutes at room temperature. The DNA from 400  $\mu$ l of supernatant was precipitated with 1.0 ml cold absolute ethanol, left at  $-40^{\circ}\text{C}$  for 20 minutes and centrifuged for 2 minutes. The pellet was dissolved in 100  $\mu$ l of a dilute salt solution containing 0.1 M Na acetate (pH 6.0) and reprecipitated with cold ethanol. The



final pellet was dried down in a vacuum dessicator and was redissolved in 40  $\mu$ l TE buffer.

Method (ii). Five hundred ml L-broth were inoculated with 2 ml of an overnight culture of the plasmid containing strain and shaken for 12 hours at the normal growth temperature. The cells were harvested, resuspended in 6 ml of a solution containing 20% sucrose in TE buffer, and after adding 1 ml lysozyme solution (10 mg/ml in TE buffer), left standing on ice for 5 minutes. 12 ml of triton-mix containing 0.1% Triton X-100, 0.04 M EDTA pH 8.0, 0.05 M Tris-HCl pH 8.0 was added and the mixture swirled gently on ice for 10 minutes to ensure complete cell lysis. The lysate was then centrifuged at 15 K rpm (8 x 50 rotor) for 45 minutes. After measuring the volume of supernatant, 0.95 g caesium chloride was added per ml to give a final density of 1.6 g/ml. 100  $\mu$ l of ethidium bromide solution (20 mg/ml) were added and the sample was centrifuged for 48 hours at 38 K rpm in an ultra centrifuge (Ti 50 rotor). Under UV illumination, the lower plasmid band was removed by side puncture with a large bore syringe needle (G19) and the ethidium bromide was removed from the collected sample by three extractions with iso-amyl alcohol, followed by dialysis at 4°C against a buffer containing 0.05 M Tris-HCl (pH 7.4), 0.005 M EDTA 0.8 M NaCl and approximately 2 g Dowex -50 beads (acid and alkaline washed). After 4 hours the buffer was changed to TE, and dialysis was continued for a further 8 hours.

Method (iii). For growing cells harbouring resistance plasmids 1 litre L-broth containing the relevant antibiotic for plasmid selection was used. In the case of strain PAW1, or Pseudomonas



strains harbouring RP4-TOL plasmids the cells were grown in 1 litre of minimal medium containing m-toluate as carbon source. After harvesting the cells, grown overnight, they were resuspended in 30 ml, 20% sucrose in TE buffer. 12 ml 0.25 M EDTA containing lysozyme (2 mg/ml) were added and the cells were swirled gently on ice for 5 minutes. 12 ml 20% SDS (in TE) ~~were~~ then added and, after mixing by gentle ~~in~~version of the tube, the mixture was subjected to 8 x 15-second heat pulses at 60° with intermittent gentle mixing. To this, 12 ml 3 M NaOH (freshly prepared) ~~were~~ added and mixed carefully for 3 minutes so that the pH rose to 12.0-12.3. Saturated Tris-HCl buffer (pH 7.0) was then added to bring the pH of the mixture down to 8.5-9.0. It normally took 15-20 ml of the buffer and several minutes of careful mixing to achieve this. Addition of 15 ml 20% SDS (in TE) followed by 30 ml NaCl (5 M) to the mixture formed a heavy white precipitate and the tube was left standing on ice for at least 6 hours with occasional mixing. After centrifugation at 10 K rpm (6 x 250 rotor), the supernatant was decanted into a measuring cylinder and  $\frac{1}{4}$  of the volume of a solution of polyethylene glycol 6000 (50% in H<sub>2</sub>O) was added, swirled gently and left at 4°C overnight. This mixture was then centrifuged at 5 K rpm for 5 minutes (6 x 250 rotor) and the pellet was resuspended in 5 ml TES buffer. The sample was then prepared for ultra-centrifugation as in method (ii).

B. Restriction of DNA with endonucleases. Reactions were normally carried out at 37° for 90 minutes in plastic snap-cap tubes (Sarstedt). 1 µg DNA was restricted in a final volume of 40 µl



of the appropriate buffer with one unit of restriction enzyme.

1 unit is that amount of enzyme required to digest 1  $\mu\text{g}$  of DNA to completion in 1 hour at  $37^{\circ}\text{C}$ . The reactions were terminated by heating the reaction mixtures at  $70^{\circ}\text{C}$  for 10 minutes.

(Table 2.2 gives details of reaction conditions for each endonuclease used.)

C. Ligation of restriction fragments with cohesive ends. The restricted DNA was diluted to give a final concentration of 5-30  $\mu\text{g}/\text{ml}$  with 10 mM Tris-HCl (pH 7.4), 0.1 M NaCl. One tenth of the final volume (usually 100  $\mu\text{l}$ )  $T_4$  ligation buffer X10 (see Materials 2.1) was added together with the required amount of  $T_4$  DNA ligase (1 unit/ $\mu\text{g}$  DNA). Incubation was normally for 16 hours at  $10^{\circ}\text{C}$  and ligated samples were then used directly to transform competent cells.

D. Transformation of E.coli with plasmid DNA. Transformation was carried out according to the method of Humphreys et al (1979) which is as follows. A 2 ml overnight culture of ED8654 was diluted 1:20 with L-broth, grown with shaking to a cell density of  $2 \times 10^8$  cells/ml, harvested by centrifugation at 5 K rpm (8 x 40 rotor) at  $4^{\circ}\text{C}$  for 5 minutes and resuspended in 10 ml cold 10 mM  $\text{CaCl}_2$ . These cells were respun under the same conditions and the pellet was resuspended in 2 ml 100 mM  $\text{CaCl}_2$  and left to stand on ice for 60 minutes. 1  $\mu\text{g}$  of DNA was added to 0.2 ml of these competent cells and the final volume adjusted to 0.5 ml by the addition of the required amount of cold 100 mM  $\text{CaCl}_2$  solution. After incubating on ice for 45 minutes, the mixture was heat



shocked at 42°C for 3 minutes, added to 0.5 ml pre-warmed L-broth and grown with shaking for 2 hours at 37°C. Cells were then plated out onto nutrient agar containing the appropriate antibiotic to identify transformed cells. Frequencies of between  $10^3$ - $10^5$  transformed cells/ $\mu$ g DNA were normally obtained depending on the size of the plasmid, large plasmids giving lower transformation frequencies.

E. Enrichment for tetracycline sensitive transformants (Bolivar et al, 1977). Transformants harbouring derivatives of pBk322 lacking a functional tetracycline resistance determinant were enriched for as follows: 1 ml transformed cells was diluted 1:100 in L-broth containing 50  $\mu$ g/ml ampicillin and grown overnight to eliminate non-transformed cells. Two ml of this culture were used to inoculate 200 ml L-broth and these cells were grown at 37°C for 60 minutes. Tetracycline was added (4  $\mu$ g/ml) and the culture was shaken for a further 60 minutes at 37°C. Cycloserine was added (100  $\mu$ g/ml) and the cells were shaken for 2-5 hours at 37°C. This treatment killed all growing cells, i.e. tetracycline-resistant cells. The culture was then harvested, resuspended in 5 ml L-broth, serially diluted to  $10^{-4}$  and plated out on nutrient agar plates containing 50  $\mu$ g/ml ampicillin. Single colonies were tested for resistance to tetracycline by patching them onto nutrient agar plates containing 20  $\mu$ g/ml tetracycline. This selection procedure yielded approximately 80-90% tetracycline sensitive clones.



F. Transfer of DNA fragments from agarose gels onto nitrocellulose filters (Southern (1975)). After agarose gel electrophoresis the gel was stained and photographed in the usual way (see below). It was then placed in a shallow tray with 250 ml denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 minutes, washed with distilled water and immersed in 250 ml neutralisation solution (1 M Tris base 3 M NaCl adjusted to pH 5.5 with concentrated HCl) for 40 minutes. After rinsing with distilled water the gel was soaked in 250 ml 2 x SSC. The nitrocellulose filter was cut to give a 20 mm overlap all round the gel and was soaked in 2 x SSC. The apparatus for the transfer consisted of a plastic seed tray 35 cm x 20 cm containing 21 20 x SSC. A glass plate supporting a blotting paper wick (4 sheets of Whatman 3 mm paper) was positioned, supported by the ends of the tray, so that the ends of the wick were immersed in the 20 x SSC reservoir. The gel was positioned on the wet blotting paper wick and plastic strips were placed along each edge of the gel to prevent contact between the nitrocellulose, which was placed on top of the gel, and the blotting paper wick. Three sheets of Whatman 3 mm paper, cut to the same size as the gel, were soaked in 2 x SSC and placed on top of the nitrocellulose filter. A 10 cm pile of dry photographic blotting paper cut to the dimensions of the gel was then placed on top of this to retain the liquid passing through the gel and the nitrocellulose. Finally a glass plate was placed on top to ensure even contact between all the components. The duration of the transfer was normally 16 hours after which the gel was restained and viewed under UV light to ensure that transfer of DNA fragments had been successful as evidenced by their disappearance. The



nitrocellulose filter was air dried at 37°C for 30 minutes and then baked in a vacuum oven for 2 hours at 80°C. This ensures permanent binding of the DNA fragments to the filter.

G. Nick translation of DNA using  $^{32}\text{P}$  labelled nucleoside triphosphate. The method following that of Rigby *et al* (1977) is described here. 10  $\mu\text{l}$  of  $\alpha$ - $^{32}\text{P}$  dCTP were dried down under vacuum. The following additions to the label were made: 1  $\mu\text{g}$  DNA (in 30  $\mu\text{l}$ ); 5  $\mu\text{l}$  nick-translation buffer (X10); cold deoxynucleotides (dGTP, dTTP, dATP) to a final concentration of 15  $\mu\text{M}$ ; 1  $\mu\text{l}$  DNase (0.02  $\mu\text{g}/\text{ml}$ ). This mixture was left at room temperature for 2 minutes and then 1 unit of DNA polymerase I was added. The final volume was adjusted to 50  $\mu\text{l}$  with sterile distilled water and the mixture was incubated at 14°C for 3-4 hours. Two drops of orange G solution (0.05% w/v) were added to the nick translation mixtures and it was then passed through a sephadex G100 column eluted with TNE buffer to separate the unincorporated nucleotides from the labelled DNA. The specific activity of the  $^{32}\text{P}$  labelled DNA was normally in the range  $10^6$ - $10^7$  cpm/ $\mu\text{g}$  DNA, which amounted to 50% incorporation of the label.

H. Hybridisation of  $^{32}\text{P}$  labelled DNA to DNA bound on nitrocellulose filters. The nitrocellulose filters were placed in plastic bags and soaked in hybridisation buffer (50% Formamide, 0.1% SDS in 2 x SSC) for 10 minutes. All the surplus liquid was then removed from the plastic bag. The labelled probe DNA was denatured by boiling for 5 minutes and immersing it into a bath of ice/water mixture so as to prevent any reannealing of DNA strands. The probe (approximately 1 ml) was mixed with 1 ml of



hybridisation buffer and added to the plastic bag. The bag was then sealed using a Calor plastic bag sealer. Hybridisations were carried out for 48 hours at 37°C with agitation. After this time the filters were subjected to 2 x 2-hour washes in hybridisation buffer followed by 2 x 1-hour washes in 2 x SSC. All washes were at 37°C with constant gentle agitation. Filters were then air dried at 37°C.

I. Autoradiography was carried out at -70°C using Dupont Cronex lightning-plus cassette (30 cm x 40 cm) and Fuji X-ray film. Exposure times varied from 2 hours to 60 hours.

J. Terminal labelling of endonuclease generated fragments with flush ends. The combined action of the 3'→5' exonuclease and the 5'→3' polymerase activities of the Klenow subunit of DNA polymerase I allows labelling of blunt ended fragments as well as fragments with cohesive ends with an appropriate  $\alpha^{32}\text{P}$  labelled nucleotide. The restricted DNA was added to 1  $\mu\text{Ci}$   $\alpha^{32}\text{P}$  dNTP (previously dried down under vacuum), where dNTP is the nucleotide at the 3' terminal of the cleavage site. The three other deoxynucleotides were added to a final concentration of 15  $\mu\text{M}$ . One unit of Klenow enzyme was then added to the mixture and incubated at room temperature for 1 hour. The samples were then ethanol precipitated, resuspended in 15  $\mu\text{l}$  of TE buffer and, after adding 2  $\mu\text{l}$  gel loading buffer, loaded onto an 8% polyacrylamide gel. After electrophoresis, the gel was dried down under vacuum (BioRad Gel Slab-dryer) and autoradiography was carried out as previously described.

N.B. When labelling fragments after digestion with nuclease S1, two  $^{32}\text{P}$  labelled deoxyribonucleotides were used to improve the amount of incorporated label.



K. Recovery of DNA fragments from agarose gels. The freeze-squeeze method of Thuring et al (1975) was used. This had been developed specifically for extracting large DNA fragments ( $\geq 5$  kb) after separation on agarose gels. After electrophoresis the gel was stained briefly (3 minutes in 1  $\mu\text{g/ml}$  ethidium bromide solution) and visualised under UV light so that the fragments of DNA were just visible. The required fragment was then cut out of the gel with a scalpel and the slice of agarose (3 mm x 10 mm) was wrapped in a small piece of parafilm. This was placed in a freezer at  $-20^{\circ}\text{C}$  for 10 minutes after which the packaged gel slice was squeezed firmly between finger and thumb for 10 seconds. The clear liquid produced was collected in a plastic snap-cap tube. To ensure maximum recovery of the DNA, the whole procedure was repeated after the addition of 75  $\mu\text{l}$  electrophoresis buffer to the remainder of the gel slice. The collected liquid was centrifuged in the Eppendorf microfuge for 5 minutes to pellet any remaining particles of agarose. Sodium acetate (3 M pH 5.5), was added to the supernatant to give a final concentration of 0.3 M acetate. and the DNA was precipitated by adding twice the final volume of absolute ethanol, leaving at  $-70^{\circ}\text{C}$  for 10 minutes and spinning in the microfuge for 10 minutes at  $4^{\circ}\text{C}$ . The pellet was then washed in absolute ethanol, respun and dried down in a vacuum dessicator. This DNA can, without further purification, be used in restriction enzyme analysis or for  $^{32}\text{P}$  labelling of the specific DNA fragment by nick translation. It is not however of sufficient purity for ligation to other DNA molecules.



## 2.4 Gel Electrophoresis

A. Polyacrylamide gel electrophoresis was performed by the method of Maniatis et al (1975). For 8% gels, the amounts of stock solutions used were as follows.

<u>Stock solutions</u>	<u>Amounts used (ml) per 100 ml</u>
Acrylamide 37.5% w/v	21.4
Bis-acrylamide 2% w/v	13.0
10x TBE buffer	10.0
H <sub>2</sub> O	55.6

After mixing the relative amounts of stock solutions, the mixture was degassed for 3 minutes, 1 ml of 10% (w/v) ammonium persulphate solution (freshly prepared) was then added to the degassed mixture together with 15  $\mu$ l Temed (NNN'N'-tetramethylethylenediamine, Serva). This mixture was then poured into a glass sandwich (40 x 14 x 0.15 cm) with perspex spacers sealed with water agar. A 13-well slot former was positioned and 1 hour allowed for polymerisation. The slot former and bottom spacer were removed and the gel was set up in a perspex gel apparatus (Raven) with 400 ml 1x TBE buffer in each reservoir. Gels were run at 50 V overnight, dried down and autoradiographed as previously described.

B. Agarose gel electrophoresis. Horizontal slab gels varying from 0.5-1% in agarose concentration were run submerged in troughs 35 cm x 20 cm using borate electrophoresis buffer (TBE). Gels were normally run overnight at 40 volts, stained with ethidium bromide (2  $\mu$ g/ml) for 15 minutes, destained in distilled water for 30 minutes, and photographed using a trans-illuminator UV source (ultra-violet products Inc. chromatome -62



365 nm peak transmission), Ilford FP4 film and an exposure time of twelve seconds through a red filter.

## 2.5 Preparation of Restriction Endonuclease SalI from Streptomyces albus G.

The method used was essentially that of Arrand et al (1978). 20 g cells from stationary growth phase were resuspended in 30 ml buffer containing 0.01 M Tris-HCl pH 7.5, 0.01 M  $\beta$ -mercaptoethanol and sonicated for 20 pulses of 30 seconds duration each. The cells were then centrifuged at 100,000 x G for 90 minutes after which the supernatant was adjusted to 1 M NaCl. This supernatant was then applied to a Biogel 'A' (500 x 2.5 cm) column previously equilibrated with Biogel buffer (1.0 M NaCl, 0.01 M Tris-HCl (pH 7.5), 0.01 M  $\beta$ -mercaptoethanol). Sixty fractions (5 ml each) were collected and assayed for SalI endonuclease using  $\lambda$  DNA. Fractions containing peak activity of endonuclease were combined and dialysed against phosphocellulose buffer, 10% glycerol, 0.01 M  $K_3PO_4$  pH 7.4, 0.01  $\beta$ -mercaptoethanol,  $10^{-4}$  M EDTA, before being applied to a phosphocellulose column (25 x 1.2 cm) and eluted with a linear gradient (200 ml total volume) from 0-1.0 M KCl. Since an exonuclease elutes at about 0.65 M KCl and SalI at about 0.7 M KCl, the late fractions are relatively free of exonuclease. These fractions were concentrated by dialysis against a solution containing 50% PEG (polyethylene glycol) (6000), 0.1 M  $K_3PO_4$  pH 7.4, 0.01 M  $\beta$ -mercaptoethanol  $10^{-4}$  M EDTA. The specific activity of the final product was 1 unit/ $\mu$ l where 1 unit was that amount of enzyme required to digest 1  $\mu$ g of  $\lambda$  DNA to completion in 1 hour at 37°C.



## CHAPTER 3    EXCISION OF THE 40 kb SEGMENT OF THE TOL PLASMID INVOLVES DIRECT REPEATS

### 3.1    Introduction

The loss of the toluene degrading function from strains of Pseudomonas putida carrying the TOL plasmid pWWO can be selected for by growing cells on benzoate, an intermediate in both the chromosomally encoded ortho-pathway and the plasmid encoded meta-pathway. The reason for this is that cells growing on benzoate via the ortho-cleavage route outgrow those using the meta-pathway, thus imposing a selection advantage for cells which have lost the plasmid encoded function. Such loss can occur either by loss of the whole plasmid from the cell or by a specific deletion of 40 kb of TOL plasmid DNA resulting in the formation of a Tol<sup>-</sup> plasmid, the archetype of which is pWWO-8 (Bayley et al, 1977).

In Bayley et al's study, 10 independently isolated Tol<sup>-</sup> derivatives of PAW1 which had been grown on benzoate were described. Of these, six contained plasmids of a size smaller than pWWO; on restriction enzyme analysis with EcoRI the fragment patterns obtained suggested that the deletion which had occurred was identical in each case. Nine EcoRI fragments were missing from the Tol<sup>-</sup> plasmids when compared to digest patterns of pWWO DNA. The molecular weights of these fragments added up to approximately 27 Md which is equivalent to about 40 kb of TOL DNA. As no detectable levels of meta-pathway/<sup>enzymes</sup>were observed in the strain carrying pWWO-8 (PAW8), it was suggested that some, if not all, of the degradative genes were carried on this 40 kb DNA segment.

The ease and specificity with which pWWO-8 type plasmids



form from pWWO suggests the involvement of some structural feature of the plasmid. One possible mechanism for specific excision events is that of reciprocal recombination between DNA sequences in direct repeat at the ends of the excised region (the Campbell model, 1966). Examples of this are the precise excision of the F plasmid from the E.coli chromosome and the excision of resistance determinants from R factors (Chandler, 1979).

The molecular basis for the excision event is the subject of this chapter and the experiments have been designed to test the hypothesis that it occurs via recombination between two copies of a directly repeated sequence occurring at both ends of the excised segment. Cloning techniques, restriction enzyme analysis and electron microscopy have been employed to determine the structure of the DNA (i) at the ends of the excised region in pWWO and (ii) at the point on pWWO-8 where the excision event has occurred. A portion of this work was initiated by Dr R.G. Downing.

### 3.2 Cloning and Analysis of HindIII fragment Hd from pWWO-8

From the restriction mapping data of Downing et al (1979), and Downing and Broda (1979), it was apparent that the ends of the excised region lay in the HindIII fragments HD and HF of pWWO and that the novel HindIII fragment Hd in pWWO-8 was formed as a consequence of some site specific excision event (see Fig. 3.1).

As a first step in the structural analysis of HindIII HD and HF, they were obtained as cloned fragments in the plasmid vector pBR322 and detailed restriction maps of both recombinant plasmids (pED3306 and pED3307) were elucidated by Dr R.G. Downing. The apparent similarities between fragments HD and HF as regards the



Fig. 3.1 The excision of 40 kb of TOL plasmid DNA results in the formation of the apparently cryptic plasmid pWWO-8 from pWWO. Cells carrying pWWO-8 can be selected for by growing strains carrying pWWO on benzoate (see text for details). The ends of the excised region were shown by Downing and Broda (1979) to lie in the HindIII fragments HD and HF. As a result of this excision event, a novel HindIII fragment Hd is formed in pWWO-8.



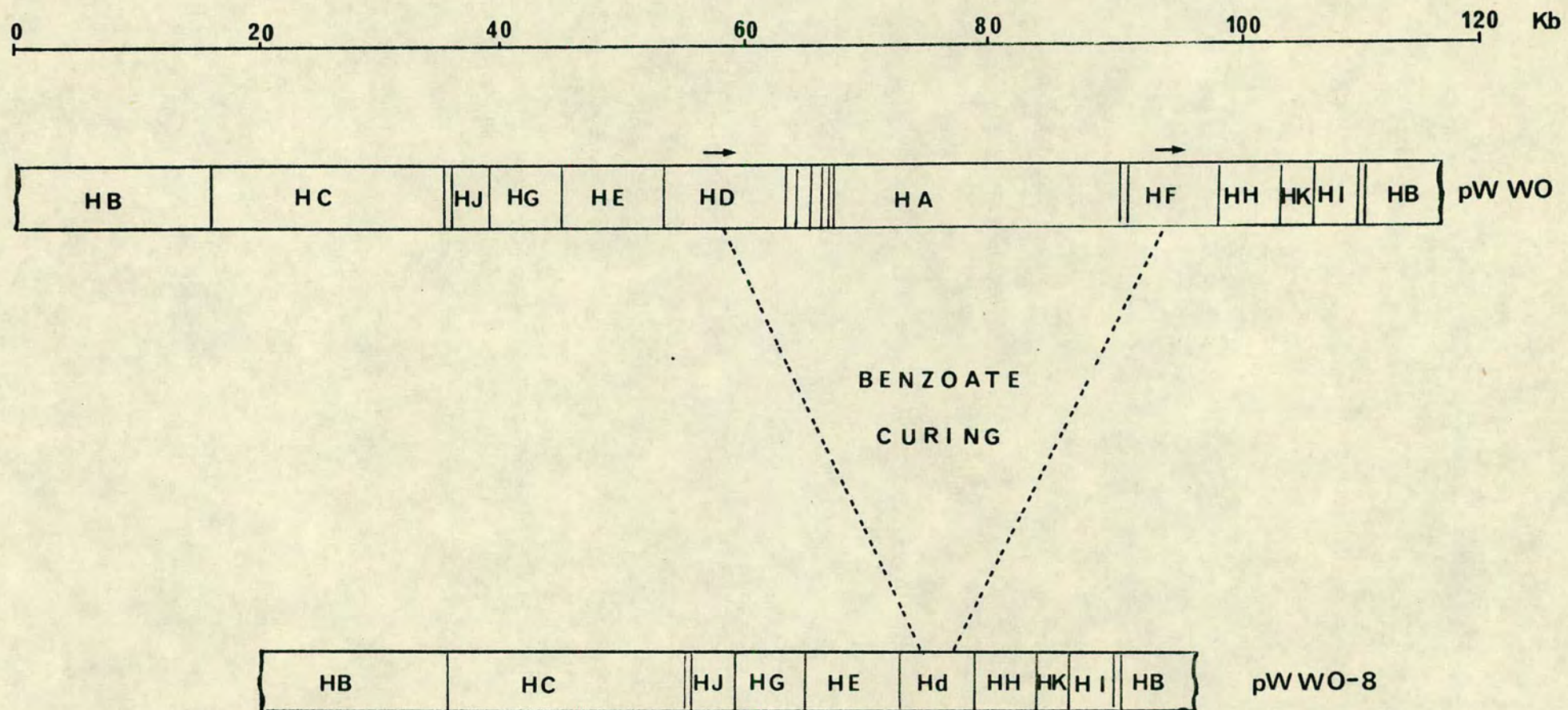


Fig 3.1



distribution of endonuclease cleavage sites prompted the analysis of the HindIII fragment Hd present in pWWO-8. The plasmid pED3308 was therefore constructed in the following manipulation.

pBR322 has a unique HindIII site within the tetracycline resistance determinant. Any HindIII fragment inserted into this site will therefore inactivate this gene. Plasmid DNA samples were prepared from ED8654 (pBR322) and PAW8, which carried pWWO-8, digested with HindIII, mixed together and ligated for 16 hours. After transformation the cells were subjected to the tetracycline sensitive enrichment procedure described by Bolivar (1977), (see Materials and Methods).

After selecting for transformed cells on ampicillin supplemented nutrient agar plates, one hundred clones were tested for tetracycline sensitivity. Ten putative clones (from 82 obtained) were screened using the Birnboim and Doly (1979) procedure. Using this method the plasmid DNA may be digested with endonucleases without further purification. Fig. 3.2 shows HindIII digests of the 10 plasmids present in the tetracycline sensitive clones tested. Comparison with the HindIII digest of pWWO in track 1 (used as a size standard) allows the individual cloned fragments to be sized. The arrow indicates the position of the required HindIII fragment Hd (which co-migrates with HF in pWWO). Track 9 contains the plasmid chosen to represent this class of recombinant and the strain from which it was isolated was named ED3308.

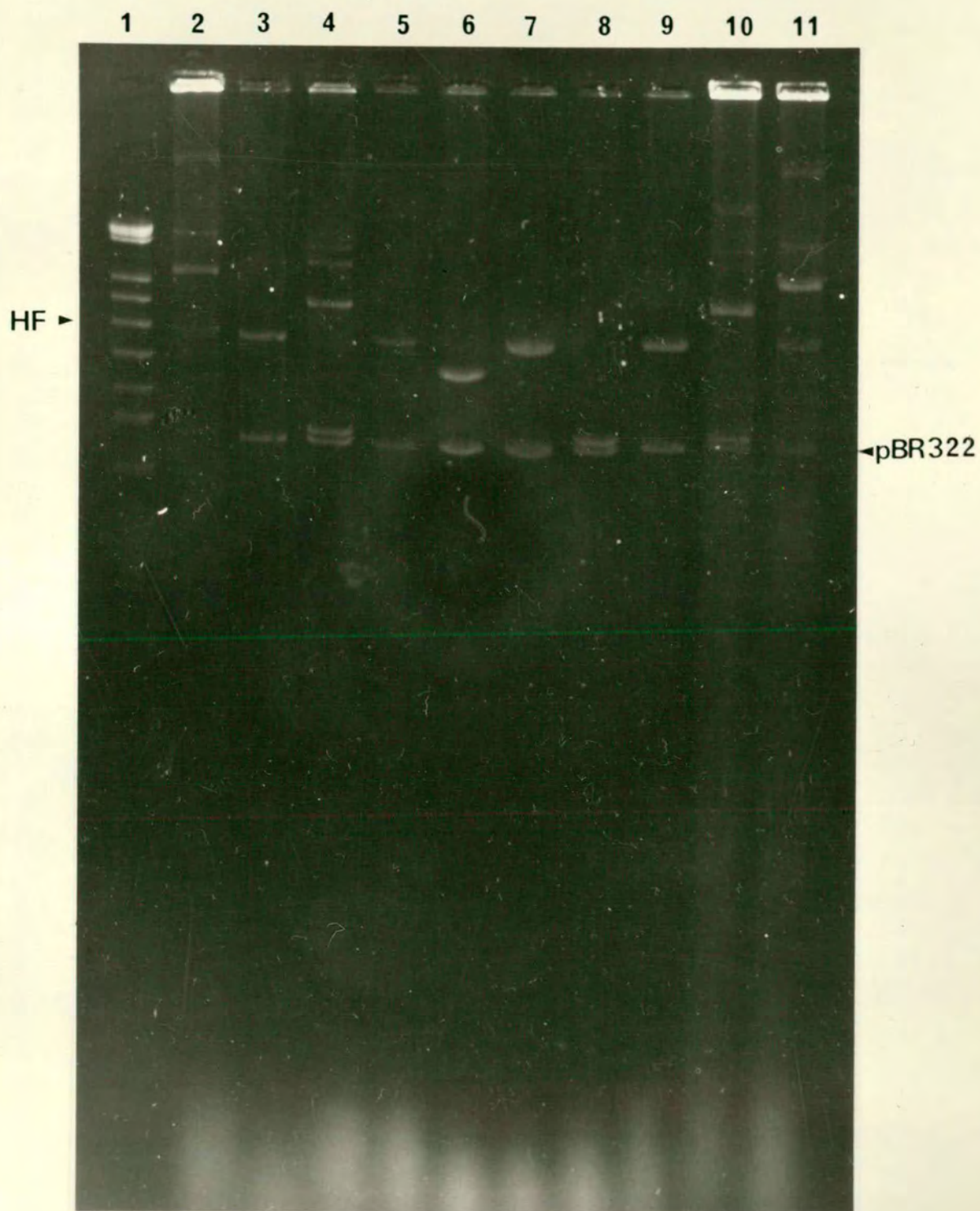
A cleavage map of pED3308 using several hexanucleotide recognising endonucleases was constructed in the following way. Fig. 3.3 shows the various restriction enzyme digest patterns which will be referred to below and the final restriction map alongside



Fig. 3.2 Screening for hybrid plasmids after a cloning experiment with HindIII restricted pBE322 and pWWO-8 plasmid DNAs. Plasmids were isolated using the Birnboim and Doly (1979) procedure, digested with HindIII and run out on a 0.7% agarose gel. Arrows show the position of fragment HF in HindIII restricted pWWO DNA (track 1) and the position of the pBR322 moiety of each recombinant plasmid (tracks 2-11). Track 9 contains the plasmid which was chosen to represent the class of recombinant in which fragment Hd was inserted into the HindIII site of pBR322 (pED3308 in this study).



Fig3.2





those of pED3306 and pED3307 is shown in Fig. 3.4. Plasmid DNA from ED3308 was prepared and digested with PstI generating three fragments and an immediate map. The unique PstI site in pBR322 is known to be 750 bp from the unique HindIII site (Sutcliffe, 1978); therefore the orientation of the cloned fragment with respect to the PstI site in the vector could be elucidated after a PstI/HindIII double digest. A single XhoI site was positioned approximately 0.2 kb to the right of the HindIII site present on the 6.6 kb PstI fragment, which is cleaved in an Xho/PstI double digest. HpaI cleaved only the 1.85 kb PstI fragment in a PstI/HpaI double digest, resulting in fragments of 1.65 kb and 0.2 kb, the latter fragment having run off the end of the gel shown in Fig. 3.3. The KpnI site was located by using data from PstI/KpnI, HindIII/KpnI and EcoRI/KpnI double digests giving the order EcoRI, PstI KpnI as shown in Fig. 3.4. The EcoRI sites on the cloned fragment were located using data from HindIII/EcoRI and PstI/EcoRI double digests.

### 3.3 Identification of a Repeated Sequence

The similarities between fragments HD and HF as regards the distribution of restriction enzyme cleavage sites are apparent (Fig. 3.4); they suggest that there is a repeat in the DNA sequence that spans the common EcoRI, PstI and KpnI sites. Since the orientation of the fragments HD and HF in the vector plasmid was obvious from the restriction mapping and their orientation in pWWO was known from the asymmetric distribution of XhoI sites, the predicted pair of repeated sequences in these fragments would lie in direct orientation in pWWO.



Fig. 3.3 (see over). Agarose gel electrophoresis of pED3308 digested with various endonucleases. Tracks denoted by S contain EcoRI cleaved pED3306 DNA which was used as a fragment size standard. Calibration graphs were constructed by plotting the reciprocal of the migration distance from the wells against the known sizes (in kb) of the EcoRI generated fragments of pED3306. The accompanying table (3.1) gives details of the endonucleases used and the fragment sizes generated. Several digestions are duplicated in the gels shown in order to clarify specific points described in the text.

Notes: In track 3 the 6.6 kb fragment is a partial digestion product.  
Incomplete digestion is also observed in tracks 6, 14, 15 and 16.



Table 3.1      Sizes (in kb) of restriction fragments shown in Fig. 3.3.      1-16 refer to the relevant tracks.

1	2	3	4	5	6	7	8
<u>PstI</u>	<u>HindIII/PstI</u>	<u>XhoI/PstI</u>	<u>HpaI/PstI</u>	<u>KpnI/PstI</u>	<u>HindIII/KpnI</u>	<u>HindIII/HpaI</u>	<u>EcoRI</u>
6.6	4.0	4.2	6.6	6.6	4.4	6.3	5.7
3.0	3.0	3.0	3.0	2.75	3.85	4.4	4.1
1.87	2.9	2.8	1.6	1.87	3.12	0.8	1.6
	1.2	1.87	0.2 (NS)				
	0.7						
9	10	11	12	13	14	15	16
<u>EcoRI/PstI</u>	<u>PstI</u>	<u>HindIII/EcoRI</u>	<u>HindIII</u>	<u>EcoRI</u>	<u>EcoRI/KpnI</u>	<u>PstI</u>	<u>PstI/KpnI</u>
4.7	6.6	4.4	7.2	5.7	5.7	6.6	6.6
3.0	3.0	4.3	4.4	4.3	4.0	3.0	2.8
1.6	1.85	1.6		1.6	1.6	1.8	1.8
1.1		1.3			0.3 (NS)		0.2 (NS)
0.8							

NS = NOT SEEN



Fig. 3.3

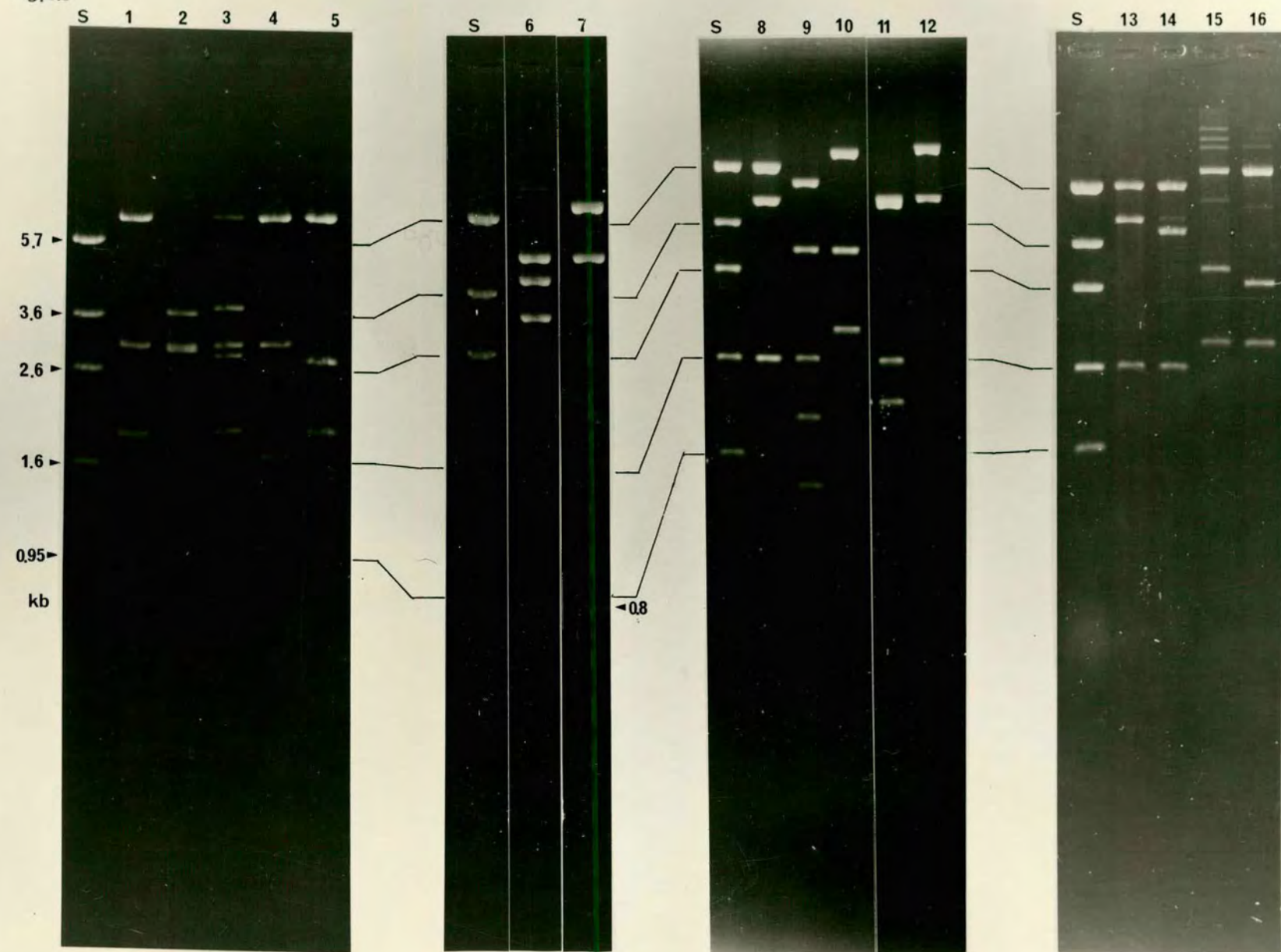




Fig. 3.4 Restriction endonuclease cleavage map of recombinant plasmids pED3306, pED3307 and pED3308. Abbreviations are as follows: H = HindIII K = KpnI R = EcoRI P = PstI X = XhoI  
B = BglII

The size and position of the region of homology spanning the common EcoRI, PstI and KpnI cleavage sites was estimated by heteroduplex analysis (section 3.3). The hatched region defines the pBR322 moiety in each recombinant.



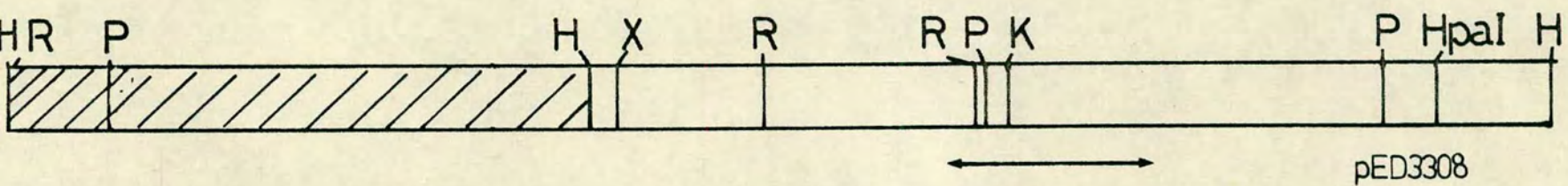
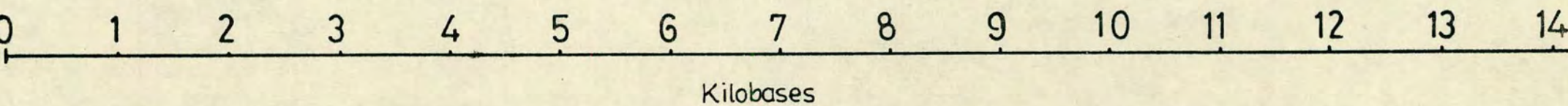
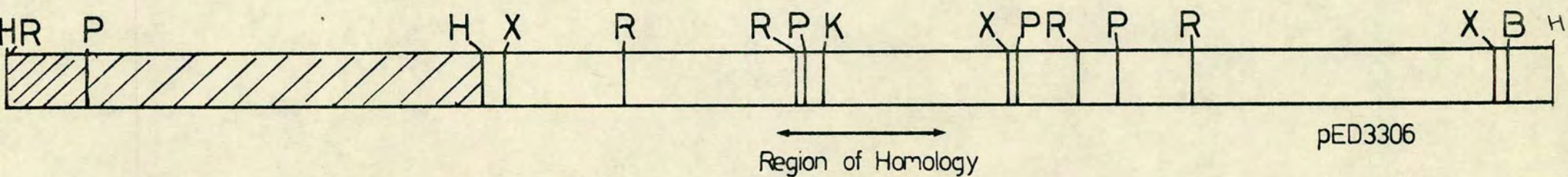
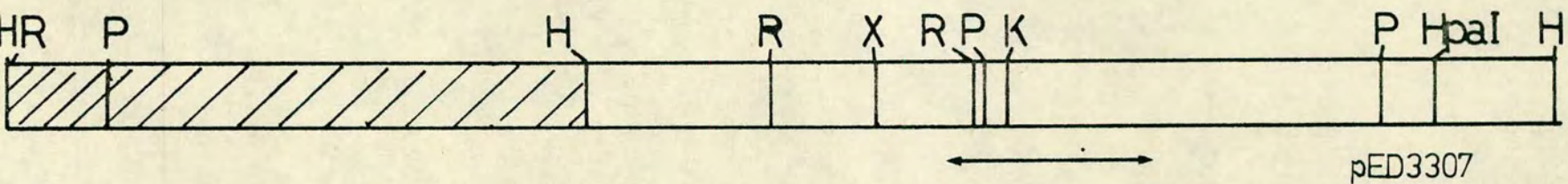


Fig 3.4





To show whether there was such a common sequence in fragments HD and HF, heteroduplex molecules were made using pED3306 and pED3307. These two plasmids were linearised by digestion with endonucleases Bgl II and HpaI respectively as these enzymes cut the appropriate plasmid at a unique site. The molecules were then denatured, mixed together and reannealed by the method of Davis et al (1971). An electron micrograph of one such molecule (of twelve measured) is shown in Fig. 3.5(a). A repeated region of 1.4 kb was revealed as being approximately 2.8 kb from the end of the pBR322 duplex on the linear molecule. This is consistent with the position of the common restriction enzyme sites, confirms the orientation of the cloned fragments in the vector plasmid and locates and sizes the repeated sequence accurately.

The pattern of target sequences on the cleavage map of fragment Hd is evidence that it contains one copy of this 1.4 kb sequence which defines the HD/HF boundary in this hybrid fragment. Confirmation of this was obtained by carrying out heteroduplex studies of pED3308 with both pED3306 and pED3307. In these experiments each plasmid was digested with HindIII before denaturation. This cut out each cloned fragment and heteroduplex molecules were thus obtained between fragments HD/Hd and HF/Hd. One of each type of molecule (of 10 measured) is shown in Fig. 3.5(b) and (c). It can be seen here that both HD and HF have homology with Hd in the relative amounts as would be predicted by the cleavage mapping.

1.4 kb is a size reminiscent of some insertion sequences found in E.coli, e.g. IS2, IS3, IS4 and IS10 (for a review, see Calos and Miller, 1980). When restriction maps of these insertion



Fig. 3.5(A) Heteroduplex molecule of pED3306 and pED3307 showing region of homology (RH). pED3306 and pED3307 were linearised with BglII and HpaI respectively before denaturation. The distance between the region of homology and the pBR322 duplex is 2.8 kb.

Fig. 3.5(B) Heteroduplex molecule of HindIII generated fragments Hd from pWWO-8 and HD from pWWO for which the sources were pED3308 and pED3306 respectively. The length of the double stranded region is as would be predicted from the restriction enzyme cleavage map (Fig. 3.4).

Fig. 3.5(C) Heteroduplex molecule of HindIII fragments Hd from pWWO-8 and HF from pWWO, the sources of which were pED3308 and pED3307 respectively, showing that both fragments are of equal size and share 4.6 kb of homologous DNA sequences.

Notes: In all cases at least ten different molecules were measured using a Ferranti Cetec digitiser linked to an Olivetti P6040 minicomputer.

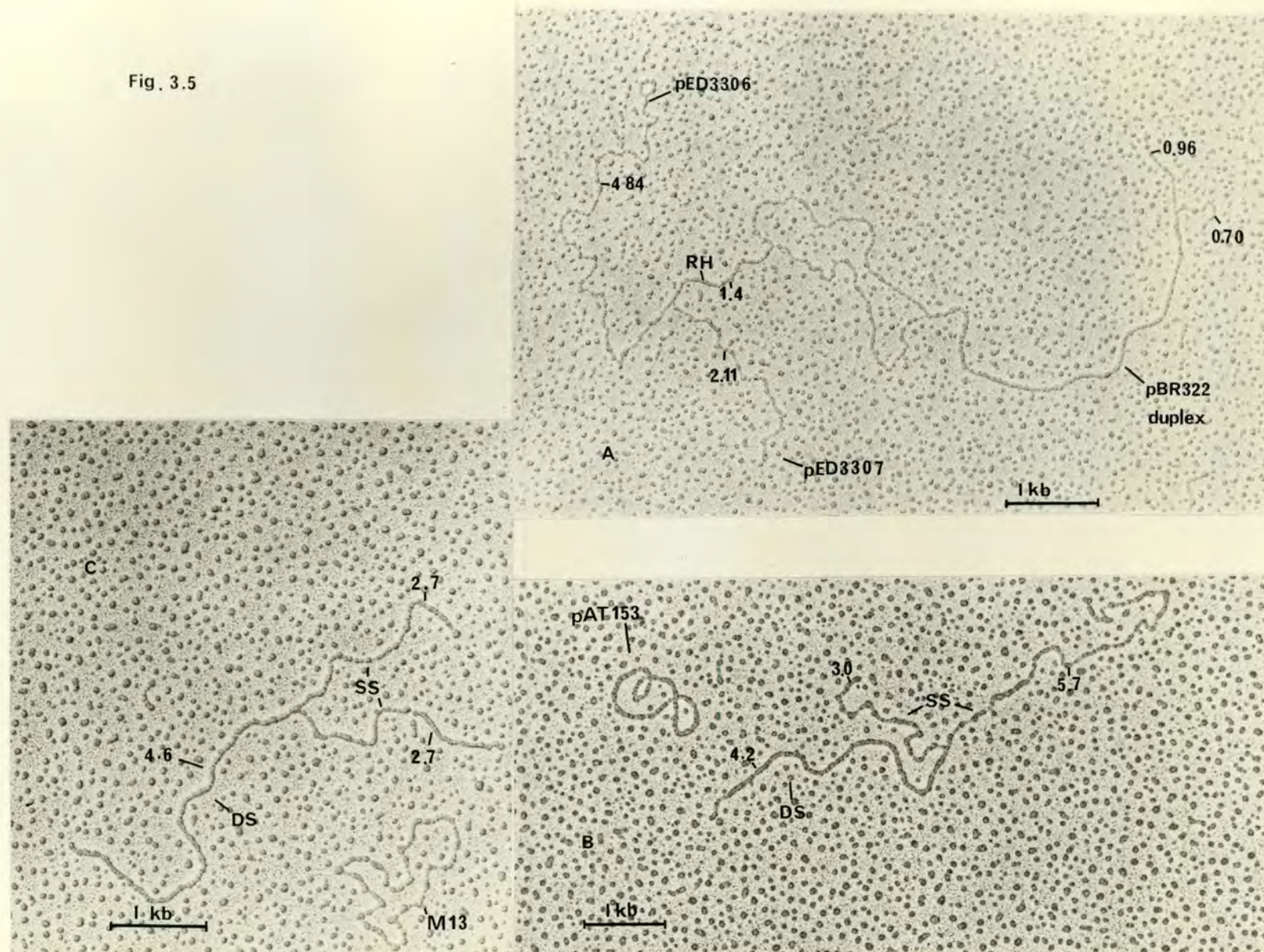
M13 (6.23 kb) was used as a single-stranded standard.

ØX174 (5.38 kb) and pAT153 (3.66 kb) were used as double-stranded standards.

All sizes are in kb.



Fig. 3.5





sequences were compared to that of the 1.4 kb sequence found in pWWO, no similarities were observed. Typical insertion sequences also have inverted repeats at the ends of the element which can form stem loop structures when single-stranded DNA molecules, containing the element, are allowed to reanneal and viewed through the electron microscope (Kopecko and Cohen, 1975). Repeated experiments of this type using the cloned fragments described above as sources of the 1.4 kb sequence, revealed no such structures (data not shown). The average size of inverted repeats at the ends of insertion sequences is about 30 bp (Calos and Miller, 1980) which may not provide a sufficient duplex region, after single-strand reannealing, for the visualisation of stem-loop structures.

Another experiment was therefore devised in which reannealed single-stranded DNA from pED3306 and pED3307 was digested with  $S_1$  nuclease, the rationale being that any duplex region formed would be protected, as  $S_1$  will only digest single-stranded DNA. If this protected fragment was then end-labelled, it should have been visible on an 8% polyacrylamide gel. Several attempts of such an experiment failed although fragments of 50 bp and 20 bp were visible in a track containing an end-labelled AluI digest of pBR322 run on the same gel (data not shown).

### 3.4 Discussion

This study has demonstrated that the specific excision event observed by Bayley et al (1977) occurs due to a reciprocal recombination event involving 1.4 kb direct repeats in the DNA sequence at the ends of the excised region. This excision results in the formation of a pWWO-8 type plasmid from pWWO and by analysing the



novel HindIII fragment Hd generated as a result of this event, it is apparent that fragment Hd is a hybrid of HindIII fragments HD and HF of pWWO. That one copy of the 1.4 kb repeated region defines the HD/HF boundary has been shown by heteroduplex analysis of the HindIII fragment Hd with both HF and HD. These observations are borne out in the similarities between the restriction enzyme target sites which have been mapped in all three fragments. Attempts to demonstrate terminal inverted repetitions in the DNA sequence, indicative of insertion elements, have failed.

Chakrabarty et al (1973) have shown that the OCT plasmid dissociates into three separate replicons when transferred to P.putida from its natural host P.oleovorans. Mechanisms involving recombination between repeated sequences, similar to that described above, may explain these observations. Chakrabarty et al (1978) also described the dissociation of the TOL plasmid in P.aeruginosa strain PAO. In this case two replicons were reported to be present in this strain, TOL $\Delta$  an 80 kb plasmid which was transmissible and could mobilise the second replicon TOL\*, a non-transmissible 40 kb plasmid which encoded the TOL degradative function. It is indeed noteworthy that of the tol genes mapped so far, all are located within the 40 kb segment which is lost after benzoate selection. Further evidence to support the idea that all of the degradative genes are located between the 1.4 kb repeated sequences was provided by the analysis of some Tn401 transposon insertion derivatives isolated by Benson and Shapiro (1978). Those insertions giving a Tol<sup>-</sup> phenotype, which had not promoted deletions of the TOL plasmid, all mapped within the 40 kb excised region (R. Downing and P. Broda, personal communication).

The dissociation of the TOL plasmid as envisaged by Chakrabarty



et al (1978) has not been reported elsewhere. Moreover, White and Dunn (1977) have described the instability of the TOL plasmid in Pseudomonas aeruginosa strain PAO in some detail but failed to correlate this with plasmid dissociation.

It is striking that the structure of the TOL plasmid resembles that of certain drug resistance plasmids, e.g. R100, where the drug resistance determinants are separated from the resistance transfer factor by directly repeated copies of IS1 (Sharp et al, 1973). The tol genes in pWWO seem to be separated from the transfer factor (pWWO-8) by directly repeated sequences of 1.4 kb in size.

In the case of R100, the drug resistance genes have been shown to be transposable as a unit due to the flanking IS1 sequences (Meyer and Iida, 1979). After the isolation of RP4-TOL hybrid plasmids, a similar model was postulated for the excised region of the TOL plasmid carrying the tol genes (Jacoby et al, 1978). However, as yet, no evidence for the 1.4 kb sequences on pWWO being insertion sequences, and therefore promoting the transposition of the DNA between them, has been obtained. In Chapters 4 and 5 experiments are described which attempt to answer some questions regarding the validity of the 'TOL' transposon' theory.



## CHAPTER 4 THE ANALYSIS OF RP4-TOL HYBRID PLASMIDS

4.1 Introduction

After the in vivo isolation of hybrids involving TOL and various resistance plasmids, a number of laboratories alluded to the existence of a TOL transposon (Chakrabarty et al, 1978; Jacoby et al, 1978). In Chapter 3 it was demonstrated that the loss of the 40 kb segment from pWWO is due to the presence of 1.4 kb direct repeats in the DNA sequence at the ends of this excised region. This segment carries those tol genes which have been genetically mapped so far (Nakazawa et al, 1980; Franklin et al, 1981) and so could be a candidate for a translocatable DNA segment carrying the toluene degradative pathway.

Reports of TOL interacting with resistance plasmids are widespread and hybrid plasmids have been constructed 'in vivo' between TOL and RP4 (Chakrabarty et al, 1978; Jacoby et al, 1978; Nakazawa et al, 1978; Franklin and Williams, 1980), R91 (White and Dunn, 1977), R702 (Chakrabarty et al, 1978), pMG18 and R2 (Jeenes and Williams, 1981) and pMG5 (Jacoby et al, 1978).

In this study, six independently isolated RP4-TOL plasmids have been analysed in order to test the 'TOL transposon' theory. These plasmids are suitable for structural analysis, as RP4 is both genetically and physically well characterised. A brief description of RP4 is therefore given here.

RP4 was first isolated from Pseudomonas aeruginosa by Datta et al (1971), is identical to the plasmids RP1, R68 and RK2 (Burkhardt et al, 1979), and is a member of the incompatibility group IncP-1. RP4 has a size of 56 kb, is transmissible throughout



a wide host range and carries resistance determinants to the drugs tetracycline, kanamycin and ampicillin (Grinsted et al, 1972).

Physical and genetic maps have been elucidated for RP1/RP4 (Barth and Grinter, 1977; Grinsted et al, 1977) and agree with those of R68 (Riess et al, 1980) and RK2 (Meyer et al, 1977). A cleavage map of RP4 is given in Fig. 4.1 showing the positions of various genetic determinants referred to in the text in this Chapter and in Chapter 5.

Cointegration events occur readily with RP4 and other replicons as evidenced by the fusions between RP4 and the large Ti plasmids of Agrobacterium tumifaciens. The formation of these cointegrates is mediated either by TnA (Hooykaas et al, 1980), or by the DNA sequence named IS8, which is located next to the kanamycin resistance determinant (Depicker et al, 1980). In both cases the mechanism by which fusion occurs is thought to be by the formation of a cointegrate intermediate during transposition of either TnA or IS8 from RP4 to the Ti plasmid. The resulting cointegrate contains two copies of the transposable element positioned at each RP4/Ti plasmid DNA junction. RP4 can also integrate into the chromosome of P.aeruginosa via TnA in a similar manner (Haas et al, 1981). Thus, in fusions between RP4 and other replicons, TnA and IS8 are the most likely mediators of such events.

The construction of RP4-TOL plasmids has been well documented, (Chakrabarty et al, 1978; Jacoby et al, 1978; Nakazawa et al, 1978; Franklin and Williams, 1980) and the two methods employed in their construction will be described here.

Jacoby et al (1978) isolated an RP4-TOL plasmid while testing the incompatibility characteristics between RP4 (IncP-1), and the



Fig. 4.1 Genetic and physical map of RP4.

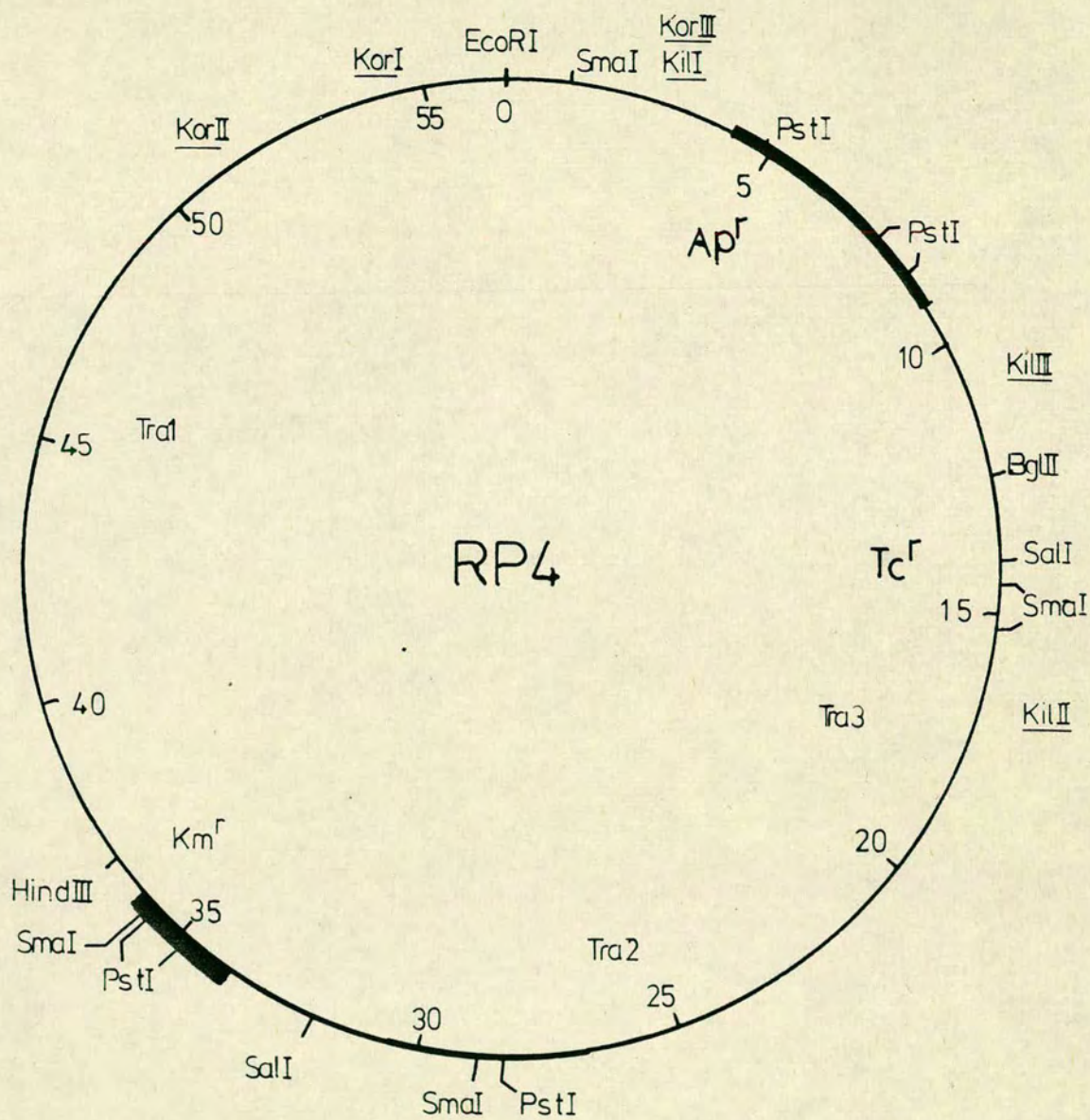
Only the relevant endonuclease target sites are shown.  
The two elements mediating replicon fusions are denoted by heavy lines. The Kil and Kor functions are referred to in Chapter 5.

After Grinstead et al 1972; Barth and Grinstead 1977; Grinstead et al 1977

Ruess et al 1980; Meyer et al 1977; Barth et al 1978



Fig4.1





TOL plasmid (IncP-9). In a series of mating experiments designed to observe the segregation kinetics of the two plasmids in P.aeruginosa PAC, one out of twenty-six exconjugants was found to be tetracycline sensitive, while retaining the other resistances conferred by RP4, and was phenotypically Tol<sup>+</sup>. Only one plasmid species was found in this type of exconjugant and it was proposed that the tol genes had translocated into the tetracycline resistance determinant of RP4. Jacoby et al (1978) also demonstrated the retranslocation of the tol genes onto another replicon in the following way. The plasmid pMG5, which encodes resistance to sulphonamides and tobramycin among others, was introduced into a strain of P.aeruginosa carrying a transfer deficient RP4-TOL plasmid. Two out of twenty exconjugants from this strain were Tol<sup>+</sup> and exhibited only the resistance determinants of pMG5. Again one plasmid species was observed which was not characterised further.

It was proposed (Jacoby et al, 1978) that the 40 kb TOL segment, described in Chapter 3, could be the TOL transposon as its excision from pWWO forming pWWO-8 was analagous to the precise excision of transposable elements, a known feature of such DNA segments (Starlinger and Saedler, 1976). In this study three of the six RP4-TOL plasmids studied were made by the method described above. These are named pED3304, pED3300 and pED3301. pED3304 is the RP4-TOL plasmid described by Jacoby et al (1978); pED3300 and pED3301 were made by Franklin and Williams (1980) and by Franklin (unpublished) respectively in P.putida.

The alternative method by which RP4-TOL plasmids have been constructed relies on the observation that the TOL plasmid does not



replicate at 42°C (Nakazawa et al, 1978). If P.aeruginosa PAO harbouring both TOL and RP4 is grown at 42°C in the presence of m-toluate as sole carbon source, Tol<sup>+</sup> clones can be obtained in which the tol degradation genes are now carried on RP4. Both Nakazawa et al (1978) and Chakrabarty et al (1978), have isolated RP4-TOL plasmids in this way and these are named pTN2, pED3302 and pED3303 in this study.

The RP4-TOL plasmids under investigation were analysed in order to determine: (i) whether a unique segment of the TOL plasmid is involved in all cases,

(ii) the site of integration of the TOL segment on RP4.

Knowledge of these two factors could help to elucidate the mechanism by which this type of hybrid plasmid is formed.

#### 4.2 Analysis of the TOL Segments Present in RP4-TOL Plasmids

In order to estimate the amount of TOL DNA carried in the different isolates, plasmid DNA was prepared from each RP4-TOL containing strain and digested with HindIII and EcoRI. Both of these enzymes cut at unique sites in RP4 and as the cleavage maps of pWWO are known for these enzymes (Downing and Broda, 1979; Lehrbach and Broda, unpublished), the TOL specific fragments could easily be identified. Fig. 4.2a and 4.2b show the pattern of fragments obtained with each RP4-TOL plasmid after digestion with HindIII and EcoRI respectively, followed by agarose gel electrophoresis.

From these data, the segment of TOL DNA present in the RP4-TOL plasmids could be estimated. Fig. 4.3 shows that:

- (i) the TOL moieties present in the RP4-TOL plasmids are all larger than 40 kb and extend beyond the 40 kb excised region at both ends,



Fig. 4.2 Agarose gels of HindIII (A) and EcoRI (B) restricted RP4-TOL hybrid plasmids. pWWO plasmid DNA restricted with the relevant endonuclease was included as a size standard (S). HindIII restricted pWWO-8 plasmid DNA was also included on gel A (S1). Tracks 1-6 in each gel contain 1. pED3300; 2. pED3301; 3. pED3302; 4. pED3304; 5. pTN2; 6. pED3303. The lettering refers to the endonuclease generated fragments as shown in the physical map of pWWO. Fig. 4.3 summarises the data presented here.



Fig 4.2

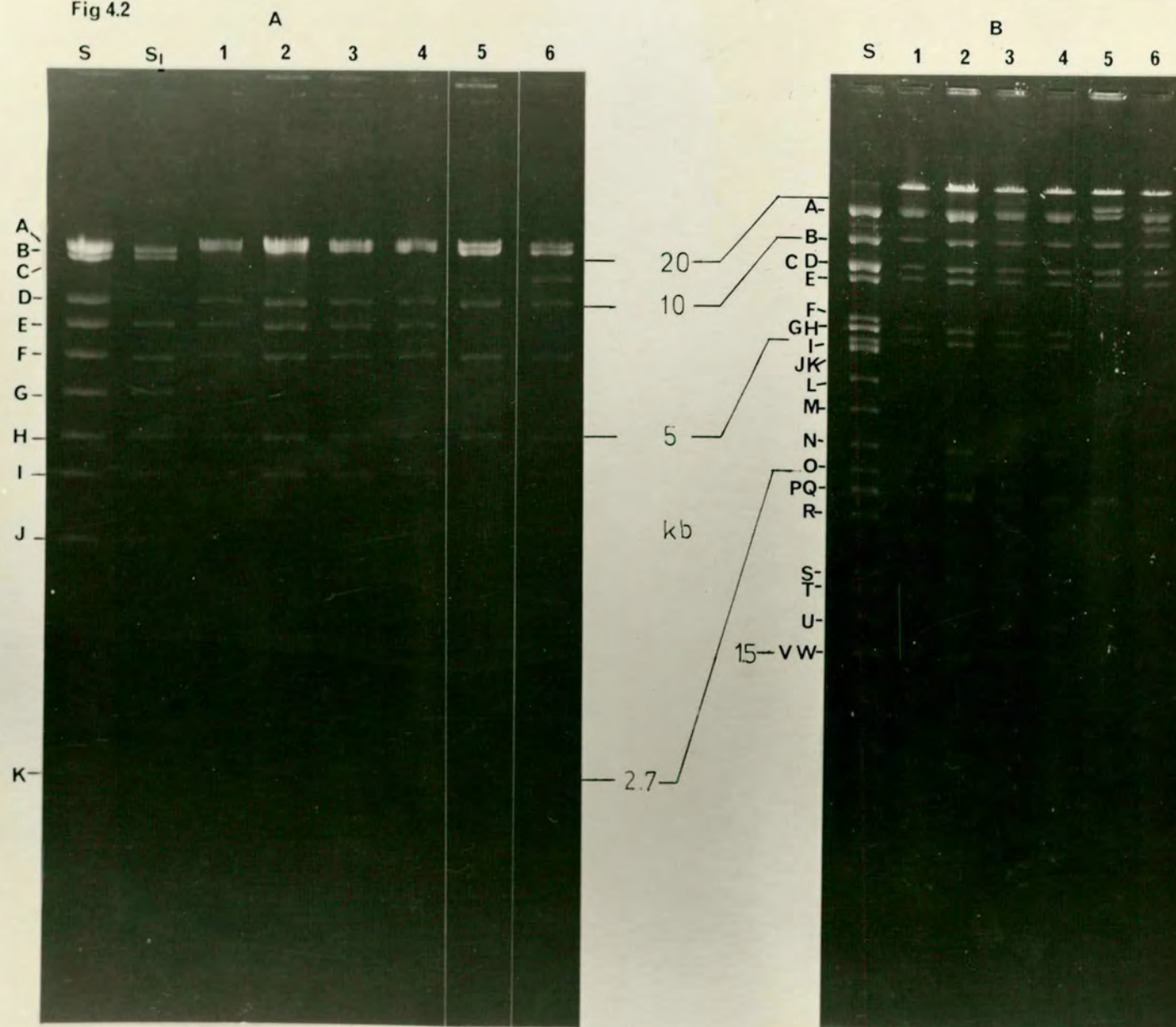




Fig 4.3

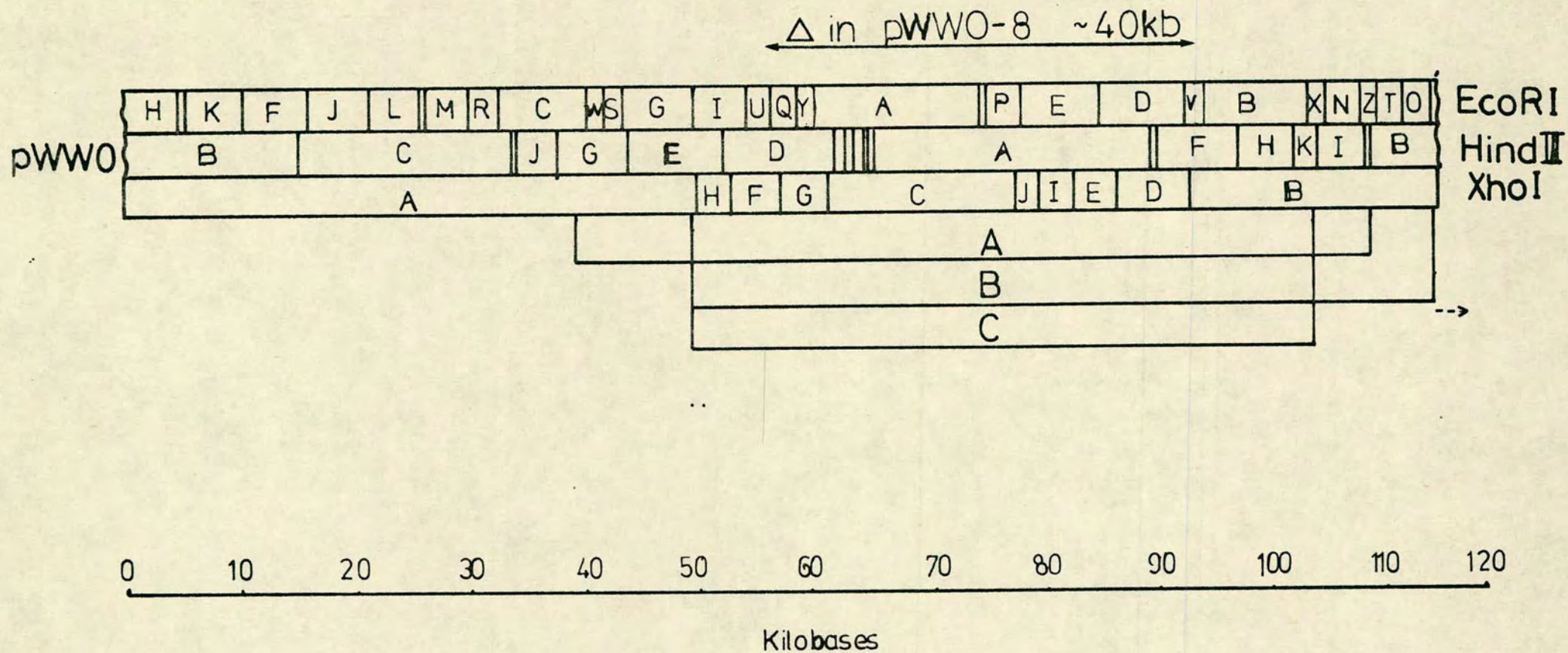




Fig. 4.3 Amounts of TOL DNA involved in the six independently isolated RP4-TOL plasmids. The extent of the 40 kb excised region is also shown. Class A includes plasmids pED3300, pED3301, pED3302 and pED3304. Class B is exemplified by pED3303 and Class C by pTN2.

Notes: In the case of pTN2, the data of Nakazawa et al (1980) ~~are~~ confirmed.

The arrow indicates that the exact end point (in EH) of segment B is unclear.

Not all of the cleavage fragments are labelled.



(ii) the amount of TOL DNA present is not the same in all cases.

In the case of those hybrids of class A (Fig. 4.3), the restriction enzyme digest patterns obtained seemed identical. One end of the TOL segment contains EcoRI fragment EW but not HindIII fragment HG, and at the other end EcoRI fragment EN but not EZ; this meant that these four plasmids contained similarly sized TOL segments  $\pm 500$  bp.

The plasmid pTN2 was shown to contain 56 kb of TOL DNA stretching from HindIII fragment HK to HE. Both EcoRI fragments EG and EI were absent (Fig. 4.2b), but XhoI fragment XH was present (data not shown), positioning one end-point of the segment in fragment HE as shown (Fig. 4.3). At the other end, EcoRI fragment EB is present but HindIII fragment HK is not, giving a very precise end-point to within 500 bp.

The analysis of pED3303 revealed that the HindIII fragment HE, and the EcoRI fragment EI were missing. As XhoI fragment XH was present (data not shown) this defined one end at a similar point to that of the TOL segment present in pTN2. At the other end however, unlike pTN2, HindIII fragments HK and HI were both present as was EcoRI fragment EO (but not EH), thus positioning the right hand end as shown in Fig. 4.3.

#### 4.3 The 40 kb Segment can still Excise from RP4-TOL Plasmids

That the 40 kb excised segment could be lost from RP4-TOL plasmids was demonstrated by the formation of the plasmid pED3305 from pED3301. PAW339 was grown in liquid minimal medium containing m-toluate as carbon source. This culture was then diluted in bacterial buffer to approximately 10 cells/ml. Twenty



tubes containing 3 ml benzoate minimal medium were inoculated with 0.1 ml of the diluted culture and grown at 30°C overnight. Ten tubes showed turbidity indicating that the other 10 tubes had not received any cells. A sample of each culture was then grown overnight in benzoate medium, diluted in bacterial buffer and plated on benzoate minimal agar supplemented with 1 mg/ml carbenicillin to select for those cells with ampicillin resistance conferred by RP4. One hundred colonies from each culture were tested for growth on m-toluate by replica plating. Approximately 10-15% of the tested colonies showed no growth on m-toluate plates. One such colony from each of the ten cultures were grown in L-broth (5 ml) overnight and plasmid DNA was isolated using the Birnboim and Doly (1979) procedure, digested with HindIII and run out on agarose gels. It can be seen from the data presented in Fig. 4.4 that all ten isolates have lost the HindIII fragments HA, HD and HF and have acquired the novel HindIII fragment Hd formed as a consequence of the excision event.

In no case did the whole integrated TOL segment specifically excise to regenerate RP4. Although not all the Tol<sup>-</sup> clones obtained were examined in this way (by plasmid screening), regeneration of RP4 would presumably also regenerate the tetracycline resistance phenotype; not one of the 1000 Tol<sup>-</sup> colonies replica plated onto nutrient agar plates supplemented with tetracycline, showed reversion to tetracycline resistance. Moreover when 10<sup>9</sup> cells were plated directly onto tetracycline enriched plates, reversion to drug resistance was not observed.

#### 4.4 An Apparent Hot Spot for the Integration of Tol DNA in RP4

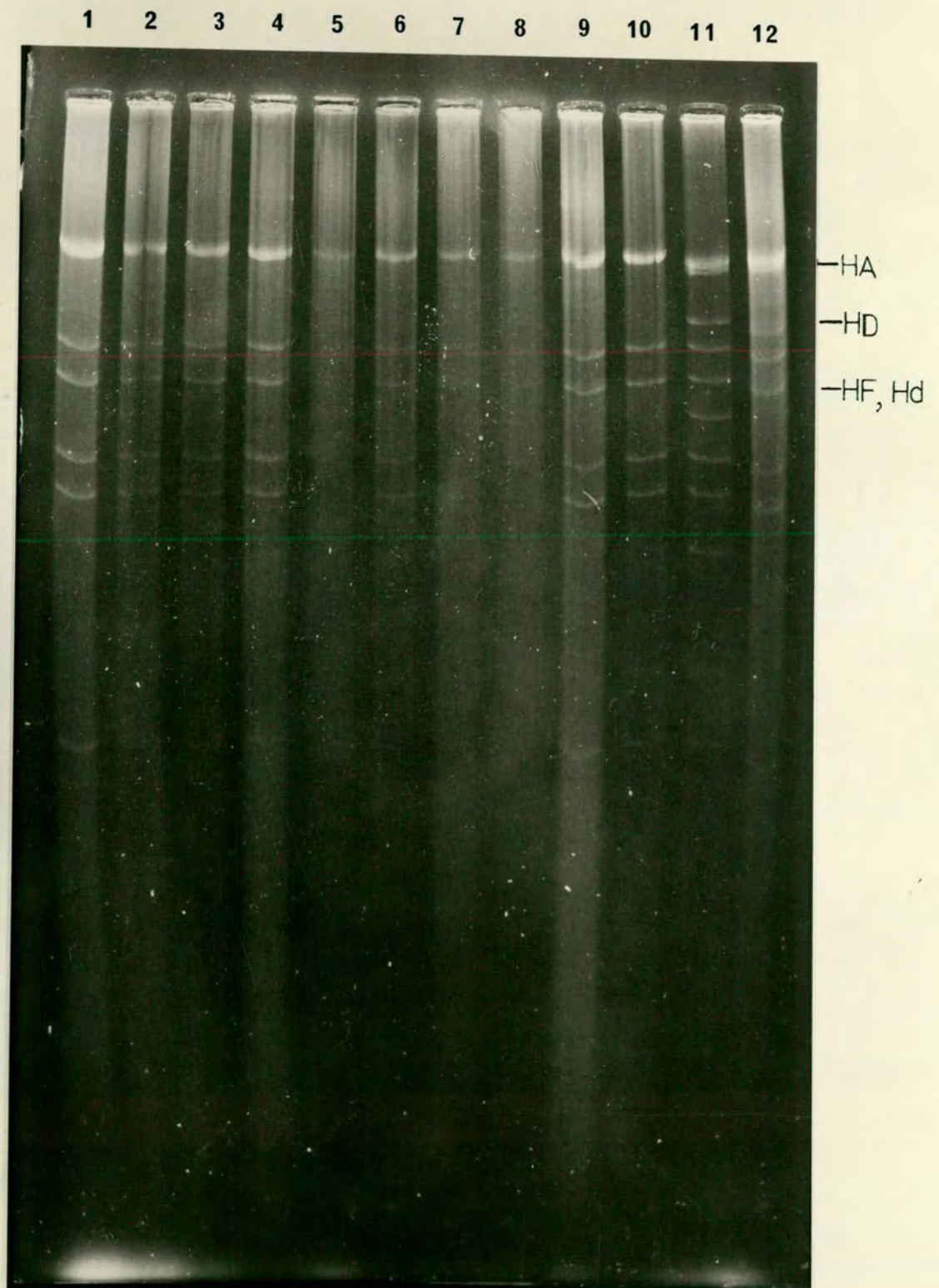
The second stage in the analysis of the hybrid plasmids was to map the site of integration of the TOL segments in each RP4-TOL



Fig. 4.4 The method of Birnboim and Doly can be usefully employed to screen for deletions in large plasmids of Pseudomonas spp. Here, ten independently isolated strains derived from PAW339 which have lost the ability to utilise meta-toluate as a carbon source have been screened for the type of plasmid they contain. After plasmid DNA isolation, HindIII digestion and agarose gel electrophoresis it was evident that all isolates contained derivatives of pED3301 (RP4-TOL) which had lost identical HindIII fragments, indicative of the specific excision of 40 kb of DNA. Track 11 contains plasmid DNA from PAW1 and Track 12 contains that from PAW339. Tracks 1-10 contain plasmid DNA from the ten Tol<sup>-</sup> isolates obtained after growing PAW339 on benzoate.



Fig 4.4





isolate. This was achieved by the inspection of restriction enzyme digest patterns on agarose gels, produced when the RP4-TOL plasmids were digested with endonucleases for which the cleavage sites on RP4 are known.

From the HindIII digest pattern of RP4-TOL plasmids of Class A (Fig. 4.2a), it was evident that two hybrid fragments (containing both RP4 and TOL DNA) of 30 kb and 26 kb (approximate sizes) were present which migrated above fragment HA (23.5 kb). The unique HindIII site in RP4 is located near the kanamycin gene and so the TOL segment must have interrupted RP4 somewhere opposite this site resulting in two large hybrid fragments of similar dimensions. This idea was supported by the observation that the hybrid fragments in the EcoRI digest (Fig. 4.2b) are approximately 40 kb and 15 kb positioning the TOL segment in this class of recombinant near co-ordinate 15 on the RP4 cleavage map (Fig. 4.1). This is consistent with the observation that such plasmids lack a functional tetracycline resistance determinant. According to the data of Barth *and Grainger* (1977) the tet gene starts at co-ordinate 14.5 on RP4. This together with the data presented in Fig. 4.5a (a SalI digest of the RP4-TOL plasmids), which shows that SalI fragment B of RP4 is altered, positions the TOL segment to within 0.5 kb as shown (Fig. 4.6).

The plasmid pTN2 when digested with SalI did not reveal any gross alteration of the SalI fragments of RP4 (Fig. 4.5a). However a hybrid fragment of 3.0 kb was present, indicating that integration had indeed occurred. Analysis of the PstI generated fragment pattern for pTN2 (Fig. 4.5b) revealed that PstI fragment B of RP4 was interrupted and a hybrid fragment of 4 kb was produced.



Fig. 4.5A Fragment pattern generated by SalI endonuclease digestion of RP4-TOL hybrid plasmids after agarose gel electrophoresis. The marker track M contains HindIII cleaved pWWO. Tracks 1-8 contain SalI cleaved. 1. pWWO; 2. RP4; 3. pED3300; 4. pED3301; 5. pED3302; 6. pED3304; 7. pTN2; 8. pED3303. The digestion was incomplete. In tracks 3-6, SalI fragment B of RP4 is altered due to the insertion of the TOL DNA segment. Fragment patterns of pTN2 and pED3303 showed little alteration of the SalI fragments of RP4, however novel fragments can be seen and are indicated by N<sub>1</sub> and N<sub>2</sub> respectively.



Fig4.5 A

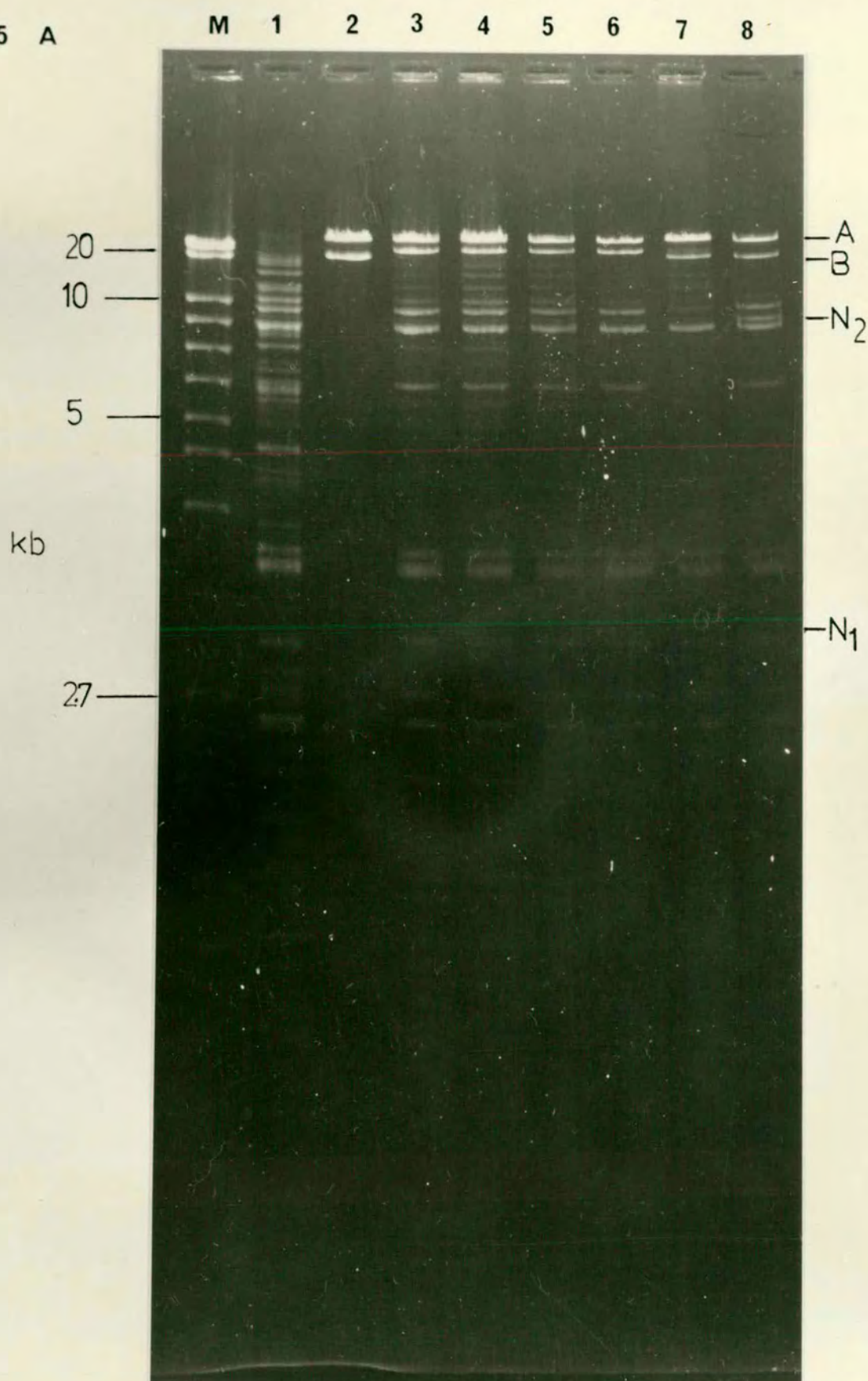


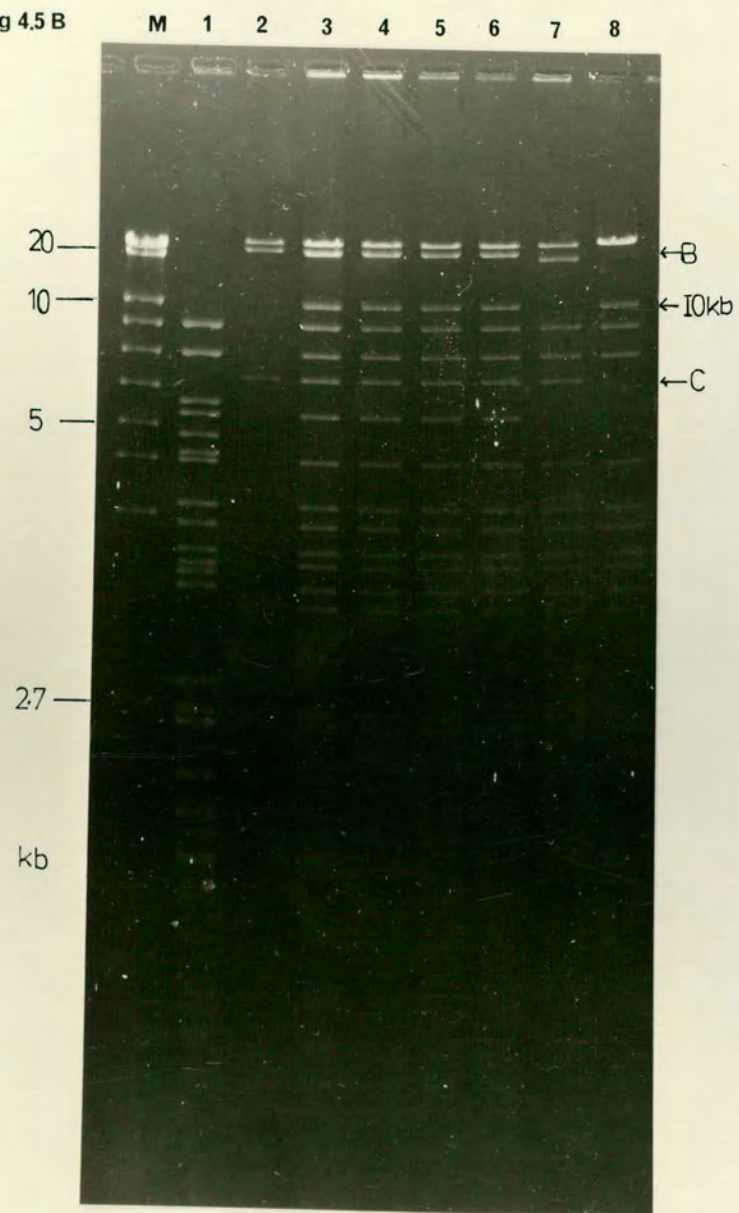


Fig. 4.5B Agarose gel showing PstI generated fragment pattern of RP4-TOL plasmids. Track M contains HindIII digest of pWWO as size marker. Tracks 1-8 contain PstI digests of 1. pWWO; 2. RP4; 3. pED3300; 4. pED3301; 5. pED3302; 6. pED3304; 7. pTN2; 8. pED3303. Positions of PstI fragments B and C of RP4 are indicated by arrows, as is the 10 kb novel fragment.

Fig. 4.5C Agarose gel showing SmaI generated fragment patterns of RP4-TOL plasmids. Tracks 1-8 contain SmaI digests of 1. RP4; 2. pWWO; 3. pED3300; 4. pED3301; 5. pED3302; 6. pED3304; 7. pTN2; 8. pED3303. The position of SmaI fragment D of RP4 (missing in pED3303) is indicated by an arrow.



Fig 4.5 B



F4.5 C

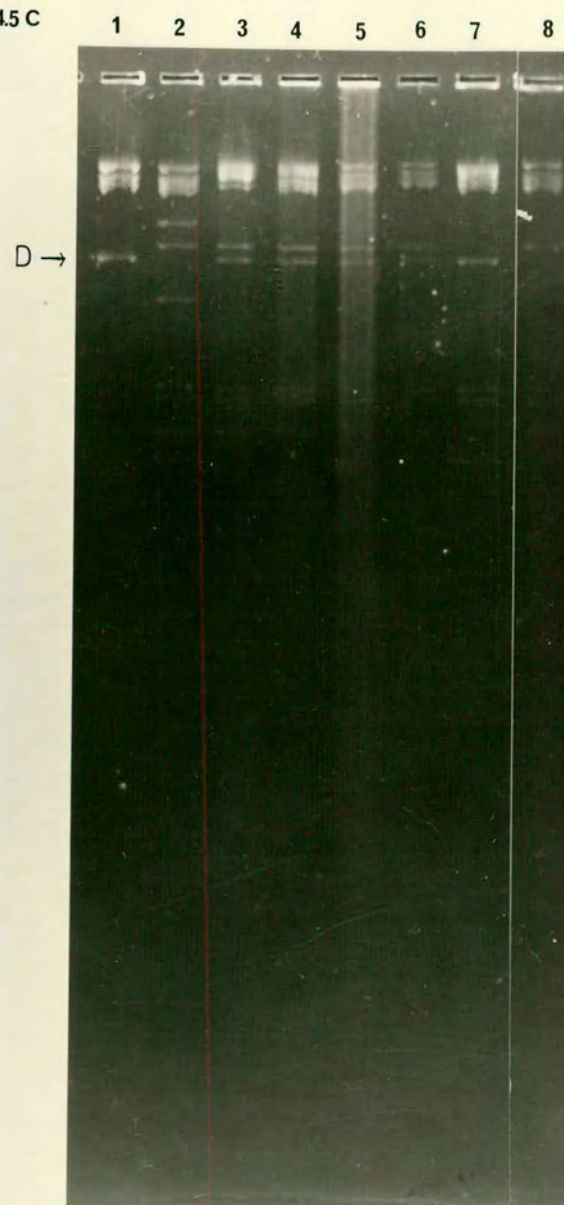


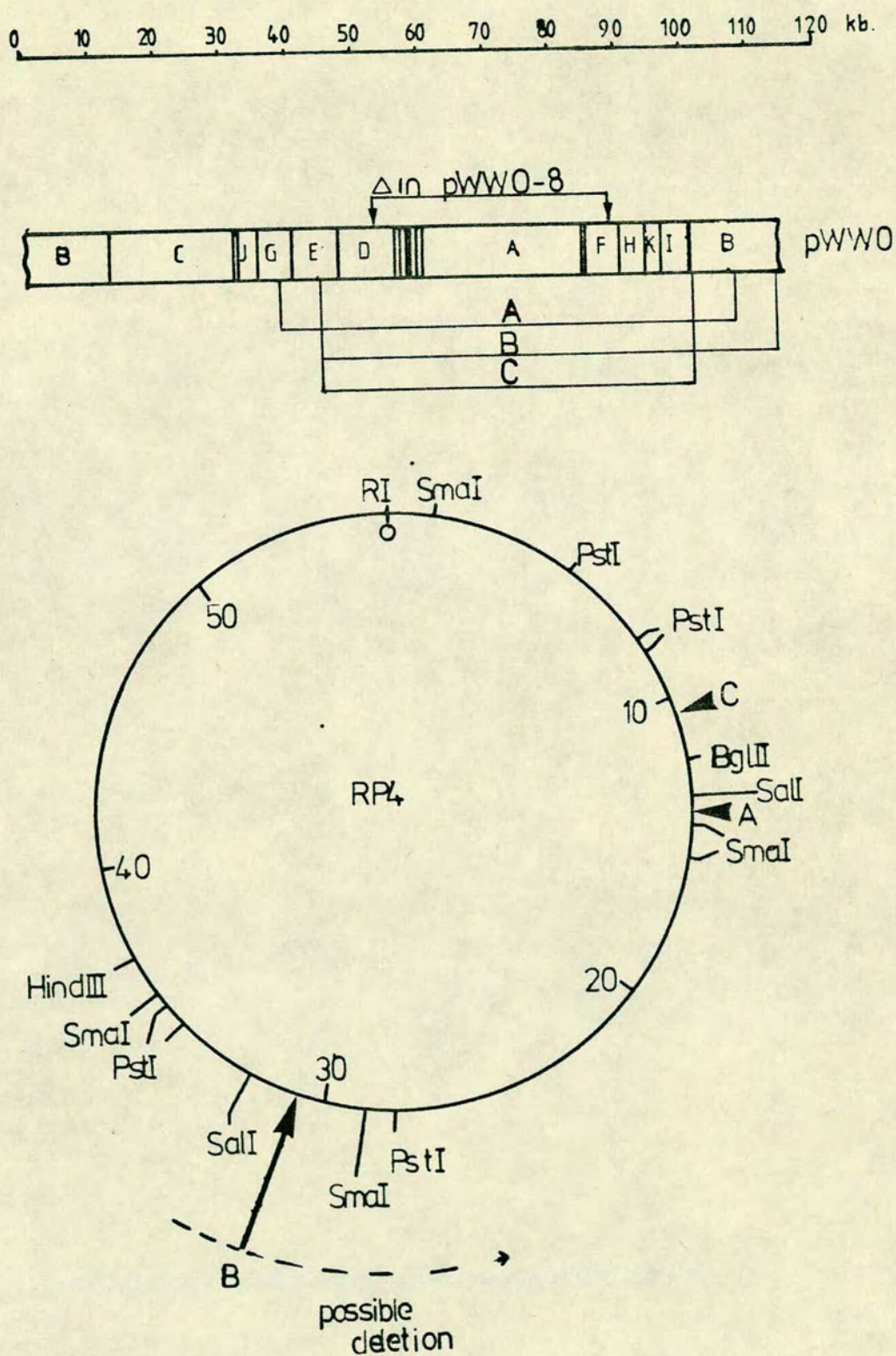


Fig. 4.6      Integration sites of the various TOL DNA segments found in the different RP4-TOL plasmids. The final positioning in RP4, of TOL segment B remains unclear (see text for explanation). Members of the different classes are as follows:

- A.    pED3300, pED3301, pED3302, pED3304
- B.    pED3303
- C.    pTN2



Fig 4.6





This together with the HindIII and EcoRI data shown in Figs. 4.2a and b position the TOL moiety in pTN2 as is shown for class C recombinant (Fig. 4.6).

In the case of pED3303 the site of insertion of the TOL segment was at first thought to be within PstI fragment C on RP4 (as it was missing); this being consistent with the observation that SmaI fragment D of RP4 was interrupted (Figs. 4.5b and 4.5c). However, inconsistent with these observations were the following: PstI fragment B was missing as such (Fig. 4.5b); an extra fragment (13 kb) appeared in the EcoRI digest pattern which could not be accounted for (Fig. 4.2b); a hybrid fragment of 14 kb was observed in the HindIII digest. The positioning of this TOL segment (B) on RP4 (Fig. 4.6) is therefore partly based on the restriction enzyme data described above together with the observation that this plasmid is transfer deficient and carries all the resistance determinants of RP4. A deletion is invoked as one possibility for explaining some of the ambiguous endonuclease digestion data. More investigations are necessary before a final conclusion is reached concerning the integration site on RP4 of this segment of the TOL plasmid. The data presented here may eventually be explained by a more complicated DNA rearrangement having occurred involving RP4 DNA.

#### 4.5 Orientation of the TOL Moieties in RP4-TOL Plasmids of Class A

Inspection of the restriction enzyme digest patterns of class A recombinants using the endonuclease PstI (Fig. 4.5b) revealed one hybrid fragment (10 kb) which was easily resolvable. This fragment (from pED3301) was isolated from a gel by cutting out the

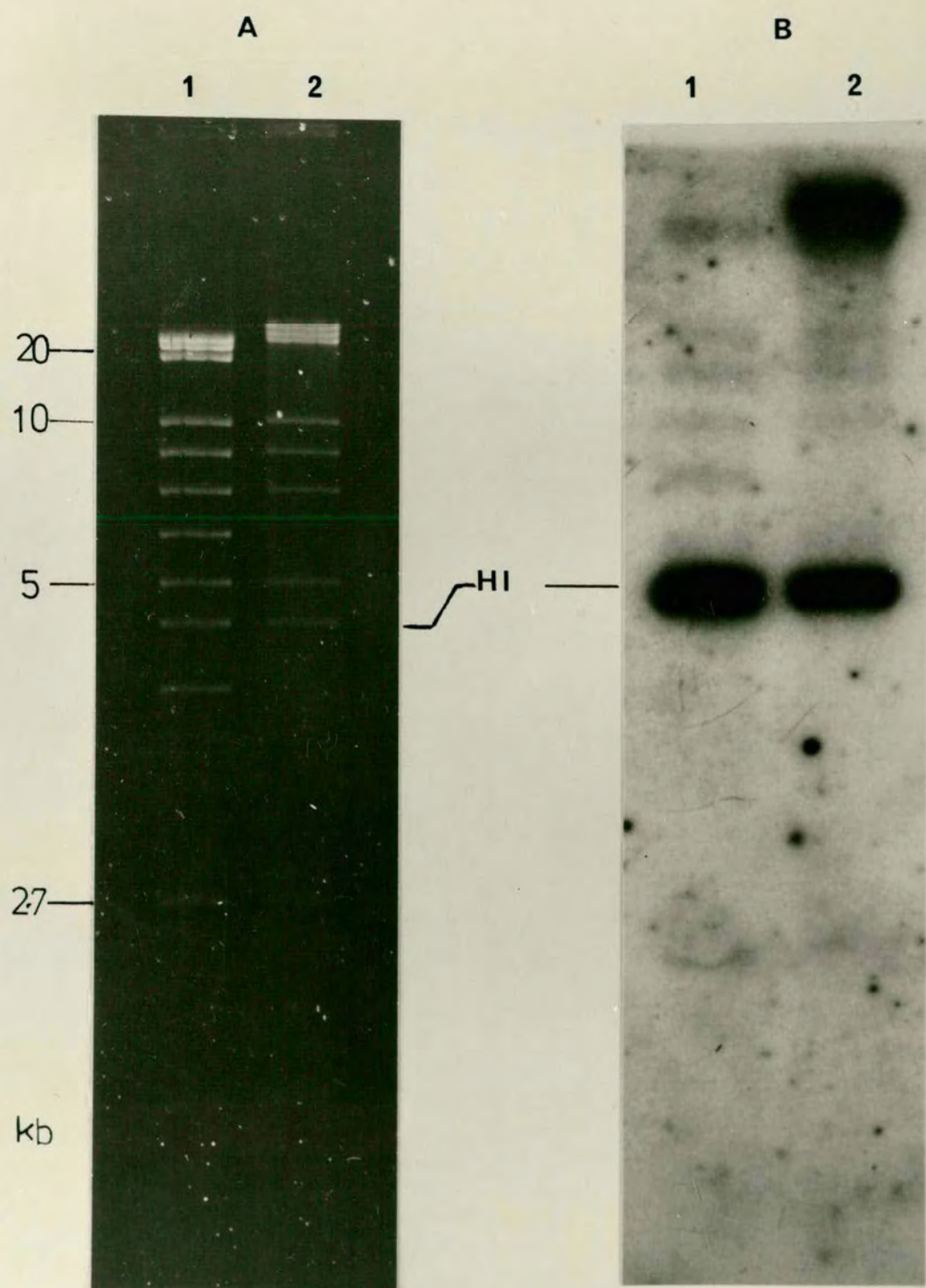


Fig. 4.7 The orientation of the TOL segment in RP4 of RP4-TOL plasmids of Class A. Tracks 1 and 2 contain HindIII digests of pWWO and pED3301 respectively. DNA fragments from (A) were transferred to nitrocellulose and hybridised against the PstI generated novel fragment (10 kb) shown in Fig. 4.5B, <sup>32</sup>P labelled by nick translation.

The overexposed autoradiogram (B) shows that only HindIII fragment HI hybridises strongly to the probe in the pWWO containing track, minimal hybridisation being observed to other fragments. This orientates the TOL moiety in these hybrids (Class A) as the PstI novel fragment (10 kb) must contain DNA sequences from the PstI site at the end of TnA on RP4 to the first PstI site in the TOL segment containing the HindIII fragment HI.



Fig 4.7





relevant slice of agarose, extracting the DNA by the method of Thuring et al (1975) and  $^{32}\text{P}$  labelling the DNA by nick translation. This probe was hybridised against Southern blots of HindIII restricted fragments of pWWO and pED3301 DNA. The result (Fig. 4.7) reveals that the probe hybridised HindIII fragment HI of pWWO which is intact in the RP4-TOL plasmids of this type. This result immediately orientated the TOL moiety in this class of recombinant as having the HI end of the TOL segment nearest the TnA element on RP4. No other HindIII fragments of pWWO showed hybridisation to the probe indicating that the end of the TOL segment must be very close to the end of HindIII fragment HI.

#### 4.6 RP4 Shares no Homology with the TOL Plasmid pWWO

One possible mechanism for the joining of two separate DNA segments is that of homologous recombination between similar or identical DNA sequences present on the two segments (Campbell, 1966). To ensure that RP4-TOL hybrid plasmids had not been formed by such a mechanism, the existence of any shared DNA sequences on RP4 and pWWO was tested in the following manner. Both pWWO and RP4 were digested with PstI endonuclease, <sup>and,</sup> after agarose gel electrophoresis, the fragments were transferred to nitrocellulose filters.  $^{32}\text{P}$  labelled pWWO DNA was used as a probe in the subsequent hybridisation. None of the RP4 fragments hybridised to the TOL DNA. Even when the X-ray film was over-exposed (the pWWO containing track turning very black) no hybridisation was observed to fragments present in the track containing RP4 (data not shown). This technique would normally pick up sequence homology of 200 bp and above.



#### 4.7 Discussion

These studies have shown that in no case is the 40 kb segment, which is excised from pWWO to form pWWO-8 type plasmids, the translocatable element involved in the formation of RP4-TOL plasmids. It is also evident that there is no unique segment of TOL DNA involved in such events as different TOL segments have been shown to be present in some of the isolates studied. Four RP4-TOL plasmids however, contained an identical segment of 65 kb of TOL DNA, at the resolution of restriction enzyme mapping. The 56 kb TOL segment found in pTN2 is similar to the TOL segment which translocates onto the resistance plasmid R2 (Jeenes and Williams, 1981) and the eight independently isolated R2-TOL plasmids described in that study had identical end-points of the TOL segment within the HindIII fragments HK and HE.

White and Dunn (1977) isolated an R91-TOL recombinant plasmid which has now been physically characterised with regard to the amount of TOL DNA present (P. Lehrbach and P. Broda, unpublished results). The segment of TOL present in this recombinant (pND3) is approximately 100 kb in size; it stretches from HindIII fragments HG to HC, and contains the 40 kb segment. The TOL segments involved in these interactions therefore exhibit variability in size and the existence of a TOL transposon consisting of a unique segment of TOL DNA is doubtful. However, there do seem to be some preferential sites on the TOL plasmid at which the translocation events described here occur. This study has also demonstrated that the excision of the 40 kb segment from RP4-TOL plasmids is still possible, although no precise excision of the whole TOL segment (a general property of most transposable elements) was observed.





The sites of insertion of the various TOL segments in RP4 also shared variability. While four of the recombinants seemed identical in this respect (class A) pTN2 and pED3303 provided the exception. The plasmids of class A all had defective tetracycline resistance determinants. Three of these plasmids were discovered because they did not confer tetracycline resistance on their hosts (pED3300, pED3301 and pED3304) and tetracycline sensitive clones were selectively picked out as those most likely to contain RP4-TOL hybrid plasmids. It is noteworthy, therefore, that the fourth member of this class of recombinant (pED3302) was made using the integrative suppression technique (Chakrabarty et al, 1978) and this fact makes a hot spot on RP4 for the translocation of the TOL segment a justifiable claim. Of the eight independently isolated R2-TOL hybrid plasmids described by Jeenes and Williams (1981), five, at least, exhibited different insertion sites of the 56 kb TOL segment in R2. However the mechanism by which those hybrids were formed may differ to that of RP4-TOL plasmid formation as R2 and TOL are known to share considerable DNA sequence homology (Bayley et al, 1979).

The possibility that RP4 could be mediating the formation of RP4-TOL plasmids could not be overlooked. It has been shown here that there is no homology between RP4 and TOL at which normal recombination could occur. The other most likely mediators of replicon interactions are IS8 and TnA. However none of the TOL <sup>e</sup>moieties map at these locations on RP4 (Fig. 4.6). The mechanism by which RP4-TOL plasmids are formed is the subject of Chapter 5.



## CHAPTER 5 THE MECHANISM OF FORMATION OF RP4-TOL HYBRID PLASMIDS

5.1 Introduction

It is evident from the conclusions reached in Chapter 4 that there is no unique segment of TOL DNA involved in the interaction between pWWO and resistance plasmids. Also, with regard to the instances involving RP4 (this study) and R2 (Jeenes and Williams, 1981) there appear to be several sites on the resistance plasmid at which the TOL segment can integrate. The insertion sites on RP4 do not correspond to the locations of either TnA or IS8 both of which have been implicated in the replicon fusions already described (Depicker et al, 1980; Hooykas et al, 1980; Haas et al, 1981). Finally, there appears to be no homology between the RP4 and TOL plasmids at which legitimate recombination could occur (Section 4.6).

The experiments described in this chapter are based on a working hypothesis that insertion sequences play a role in the formation of RP4-TOL hybrid plasmids. IS elements are known to promote the transposition of discrete units of DNA when two like elements are positioned on the same replicon (Ohtsubo et al, 1980). Many segments of DNA are transposons as a result of being flanked by IS units; for example Tn9 and Tn1681 are flanked by copies of IS1, which accounts for the mobilisation of the intervening genetic material (for a review see Calos and Miller, 1980). In all such cases the junctions between donor and recipient DNA segments are defined by a copy of the insertion element responsible for the transposition event. This fact prompted an analysis of the RP4/TOL junctions in the RP4-TOL hybrid plasmids constituting Class A (Fig. 4.6).



## 5.2 A DNA sequence is repeated at each RP4/TOL junction

Digestion of pED3305, a derivative of pED3301 which had lost the 40 kb segment (section 4.3), with the endonuclease PstI gave clearly resolvable hybrid fragments (containing both RP4 and TOL DNA) of 18 kb and 10 kb in size after agarose gel electrophoresis. The latter fragment was extracted from a gel by the method of Thuring et al (1975),  $^{32}\text{P}$  labelled by nick-translation and used as a probe against a Southern blot of PstI-digested RP4-TOL plasmids pED3300, pED3301 and pED3304. Fig. 5.1 shows the result of such an experiment, whereas the probe hybridised to a single fragment in the tracks containing RP4 or pWWO, two fragments (18 kb and 10 kb) hybridised in the tracks containing the hybrid plasmids. This hybridisation suggested that there was a repeated sequence in both RP4-TOL hybrid fragments which was not duplicated at the ends of the translocated TOL segment in pWWO.

Another explanation for the observed hybridisation could be that the site of insertion of the TOL segment in RP4 was not identical in each case. This idea was discounted for two reasons. Firstly, the same result as described above was obtained in the track containing pED3301, the plasmid from which pED3305 arose and therefore had an identical insertion site. The second reason was based on data obtained from BglII endonuclease digestion patterns on agarose gels. Digestion of pED3305 with BglII generated three fragments: 66 kb (by subtraction), 13 kb and 1.6 kb. Using the PstI generated hybrid fragment (10 kb) as a probe in a hybridisation experiment against a Southern blot of this BglII digest, all three fragments showed homology with the probe (Fig. 5.2), indicating that the distribution of BglII sites in pED3305 must be as shown in



Fig. 5.1 Autoradiogram showing hybridisation of the PstI novel fragment (10 kb) to two RP4-TOL PstI fragments in three RP4-TOL hybrid plasmids of Class A. Tracks 1-5 contain PstI digests of 1. pWWO; 2. RP4; 3. pED3300; 4. pED3301; 5. pED3304. A similar result was subsequently obtained with pED3302 (data not shown). The different ~~intensities~~ of hybridisation in different tracks may be due to unequal transfer of the DNA fragments onto nitrocellulose.



Fig5.1.

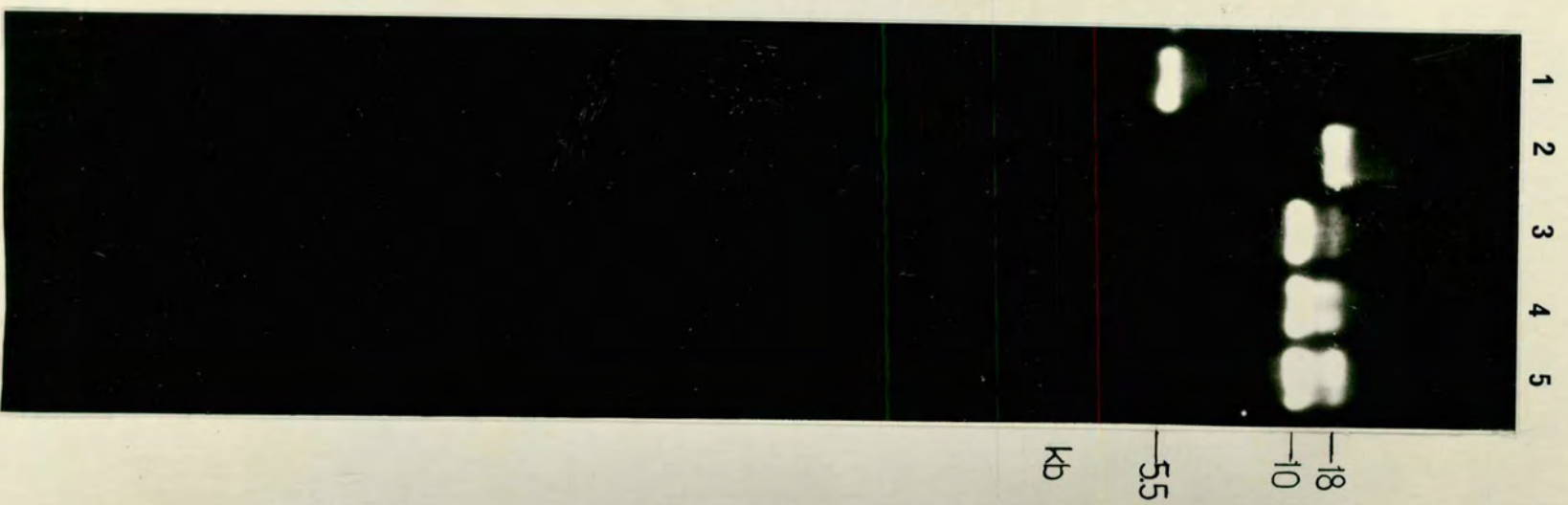




Fig. 5.2 RP4 and pED3305 were cleaved with BglII and run out on a 0.7% agarose gel (A), transferred to nitrocellulose and probed with the novel PstI generated fragment (10 kb). The result (B) shows that all three BglII fragments hybridise strongly to the probe giving the orientation of the three BglII sites in pED3305 as shown in Fig. 5.3.

Fig. 5.3 Orientation of the three BglII cleavage sites in pED3305. The heavy line denotes the extent of the TOL segment in this hybrid plasmid. Only the relevant endonuclease cleavage sites are shown. The EcoRI (RI) site is on the RP4 portion and orientates the plasmid, the two PstI (PI) sites being in TnA. The dotted line indicates the extent of the probe used in the hybridisation experiment described above in Fig. 5.2.



Fig 5.2

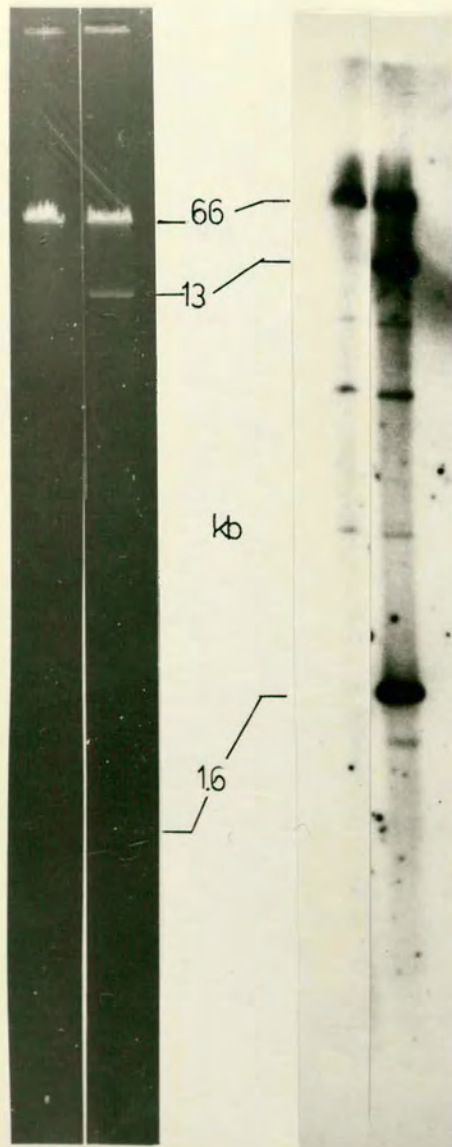


Fig 5.3

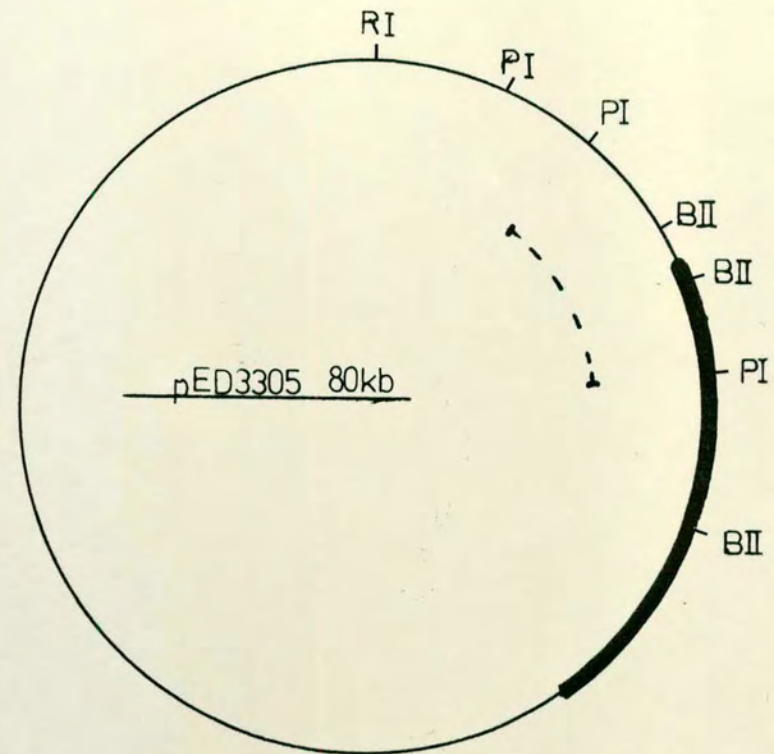




Fig. 5.3. This means that the 1.6 kb fragment is a hybrid fragment and must therefore contain the proposed repeated sequence. This 1.6 kb BglIII generated fragment was observed in the appropriate digests of all RP4-TOL plasmids of Class A (Fig. 5.4) indicating that all insertion points and TOL segments are similar (to within 100 bp) in these recombinants.

### 5.3 The origin of the repeated sequence

Since the repeated sequence did not reside on the TOL plasmid (at least in duplicated form), the possibility that its origin was from the Pseudomonas chromosome was tested by hybridising the PstI hybrid fragment to Southern blots of endonuclease digested chromosomal DNA from various strains of Pseudomonas. pED3305 was transferred from P.putida to an E.coli strain JC411; plasmid DNA was then isolated from this strain and used as a source of the PstI hybrid fragment. During plasmid DNA isolation, contamination with some chromosomal DNA is inevitable and when this DNA is subsequently  $^{32}\text{P}$  labelled, the contaminating labelled DNA may give rise to spurious results in the experiment to be described. The production of any such artifacts was therefore minimised due to (i) the specific nature of the probe (a purified fragment), and (ii) the different genetic background from which plasmid DNA was isolated.

Chromosomal DNA from three strains of Pseudomonas putida AC34, NCIB10015 and A312 and two of Pseudomonas aeruginosa PAO2 and PAC1 was isolated by the method of Dhaese et al (1979), digested with PstI endonuclease, run out on agarose gels, transferred to nitrocellulose filters and hybridised to the PstI generated hybrid fragment purified and  $^{32}\text{P}$  labelled as before. The result of this experiment (Fig. 5.4) shows that homology exists between the probe and



Fig. 5.4 Agarose gel of BglII restricted RP4-TOL plasmids of Class A including pED3305. Tracks 1-6 contain, 1. pWWO; 2. pED3300; 3. pED3305; 4. pED3301; 5. pED3302; 6. pED3304. The arrow indicates the position of the 1.6 kb fragment which is common to all RP4-TOL plasmids of this type and has been shown to be a junction fragment (section 5.2). Track M contains EcoRI digested pWWO DNA used as a size marker.



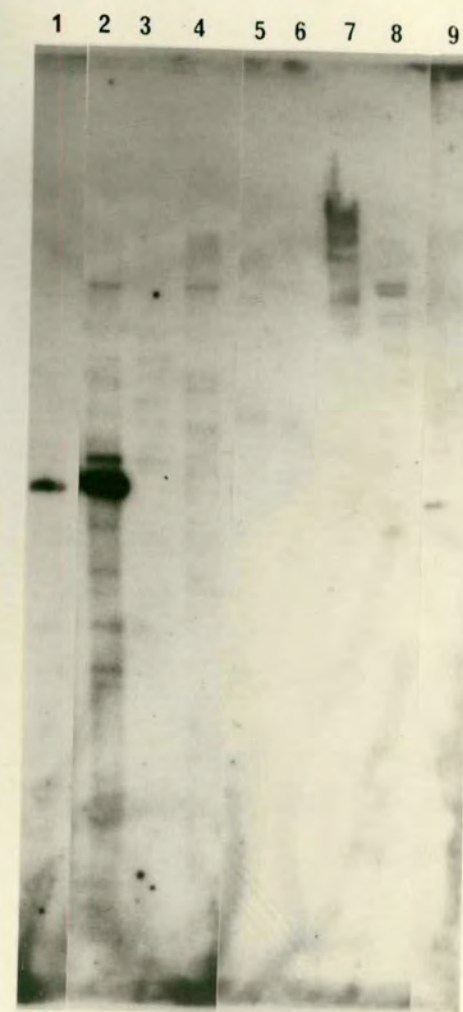
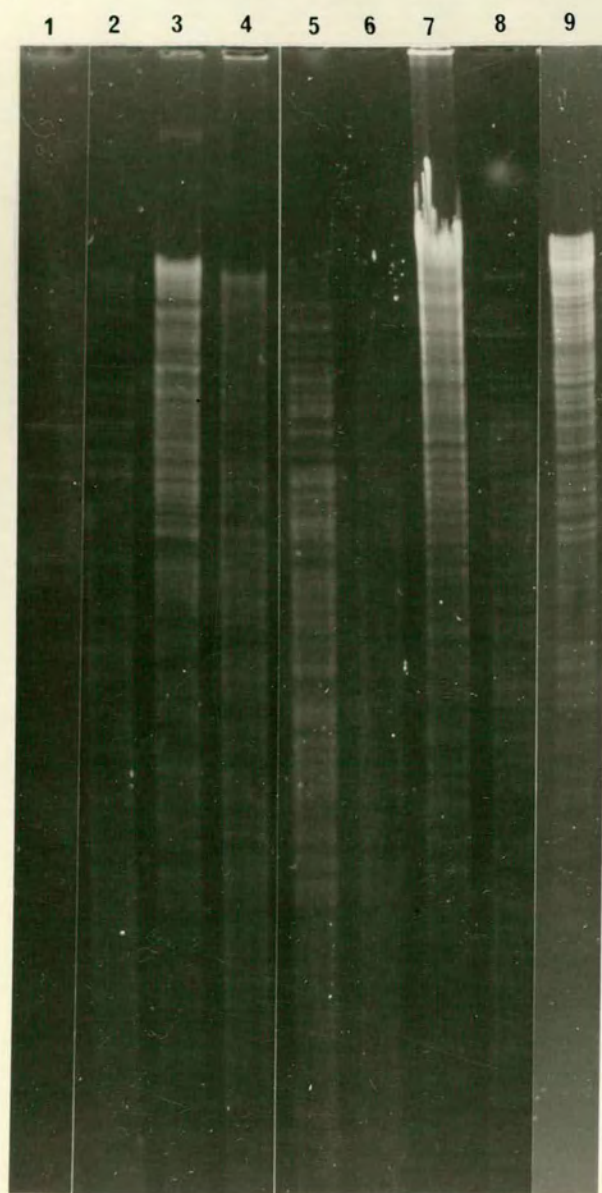




Fig. 5.5 Hybridisation of PstI novel fragment (10 kb), the source of which was pED3305, to chromosomal DNA from various strains of Pseudomonas and E.coli strain JC411. Tracks 1-9 contain PstI digests of 1. pWWO; total cellular DNA from 2. PAW1; 3. PAW82; 4. PAC1; 5. PAO2; 6. A312; 7. AC34; 8. NCIB10015; 9. JC411. Whereas homology to the probe is observed with the DNA in tracks 3, 4, 5, 7 and 8, no homology is observed with that in track 6. It should be noted that the hybridisation observed in track 9 is probably due to chromosomal DNA of JC411 contaminating the probe.



Fig 5.5





NCIB10015

chromosomal DNA of strains PA02, PAC1, and AC34 but not that of strain A312. In contrast DNA from the RP4 or TOL plasmids did not show any homology with DNA from any of these strains (data not shown).

The cloning of a hybrid fragment from pED3305 would have been useful in the characterisation of the proposed sequence. Two hybrid fragments, the 10 kb PstI fragment and the 1.6 kb BglII fragment were considered as being the most suitable for this purpose. However, no attempt was made to clone the PstI fragment as the RP4 DNA on either side of the TOL segment insertion site contains two of the three kil genes on RP4 (Figurski et al, 1981) (see Fig. 4.1). In order for DNA containing one of these functions to be cloned, its respective kor (kill override) gene must be present if the kil product is not be lethal to the cell. This however is not possible due to the distribution of these functions on RP4.

Repeated attempts to clone the 1.6 kb BglII fragment using a specially constructed vector plasmid pAB61 (kindly provided by A.M.C. Brown), which provided insertional inactivation of a <sup>\*</sup> sulphonamide resistance gene, failed although the 10 kb BglII fragment of pED3305 which contains only TOL DNA was easily obtained in the same experiments (data not shown). Further investigations are necessary before these results can be explained.

#### 5.4 A genetic test for the presence of insertion elements

The presence of insertion sequences on plasmids can be tested for on the basis that they promote the formation of cointegrate intermediates during their transposition (Ohtsubo et al, 1980). IS elements residing on a transmissible plasmid will therefore promote

\* N.B. Tests for resistance to sulphonamide were carried out using minimal medium.



the mobilisation of a non-conjugative plasmid by replicon fusion and subsequent transfer by conjugation of the cointegrate (Guyer, 1978; Willetts et al, 1981). Such a test was constructed for pED3305 as follows. pED3305 was transferred by conjugation into a pBR322-containing derivative of ED2117, a recombination deficient (recA) strain of E.coli. The small multicopy plasmid pBR322 has lost the genetic determinants which allow ColE1 to be conjugally mobilised at a high frequency by a non-recombinational mode (Warren and Sherratt, 1977; Bolivar et al, 1977). Its mobilisation from ED2117 would therefore only occur if a cointegrate was formed via an insertion element, between pED3305 and pBR322. The tetracycline resistance conferred by pBR322 was selected after mating ED2117 containing both plasmids with a recombination proficient E.coli strain ED2196. While the transfer frequency of kanamycin resistance conferred by pED3305 was  $6 \times 10^{-1}$  per recipient, no tetracycline resistant exconjugants were obtained. This result indicated that no cointegrates were formed (at least not at a detectable frequency) as pBR322 was not mobilised by pED3305. A possible explanation for this result is given in the following discussion.

#### 5.5 Discussion - A model for the mechanism of formation of RP4-TOL hybrid plasmids

One interpretation of the results of experiments described above is that there is a repeated sequence present at the RP4/TOL junction of the RP4-TOL plasmids, classified in Chapter 4 as Class A, the presence of which may give an indication of how these plasmids were formed. This sequence, which appears to have its origins in the Pseudomonas chromosome, resides on a 1.6 kb BglIII



restriction endonuclease DNA fragment and is probably smaller than 1 kb as this fragment must contain both RP4 and TOL DNA sequences. Due to its small size, it is possible that this proposed insertion element does not encode the enzymes necessary for its own transposition. This is supported by the failure to obtain cointegrates between pED3305 and pBR322 in a recA E.coli strain (section 5.4). It was interesting to note that TnA, resident on the RP4 plasmid present in pED3305, did not mediate cointegrate formation. This may be explained by the fact that the ampicillin resistance gene of pBR322 is derived from Tn3 (which is identical to TnA) and this plasmid may therefore be immune to act as a receptor replicon for a further TnA sequence (Robinson et al, 1977).

Since it is likely that Pseudomonas chromosomal genes will be required for the transposition of the proposed insertion sequence, the type of experiment described above needs to be carried out in recombination deficient Pseudomonas strains using a vector plasmid which is physically well characterised and replicates in Pseudomonas. RSF1010 is such a plasmid (Nagahari and Sakuguchi, 1978) but unfortunately is mobilised at high frequency by RP4 (Willetts and Crowther, 1981). Suitable mobilisation deficient (mob<sup>-</sup>) derivatives of RSF1010 have recently been constructed (Bagdasarian and Timmis, 1981) which would allow the stringent genetic tests described here to be carried out.

A model based on the results in Chapters 4 and 5 is presented in Fig. 5.5 and proposes one way in which RP4-TOL hybrid plasmids may be formed. This model assumes the existence of IS-like elements in the Pseudomonas chromosome and proposes that one of



Fig. 5.6     A model for the mechanism of formation of RP4-TOL plasmids.

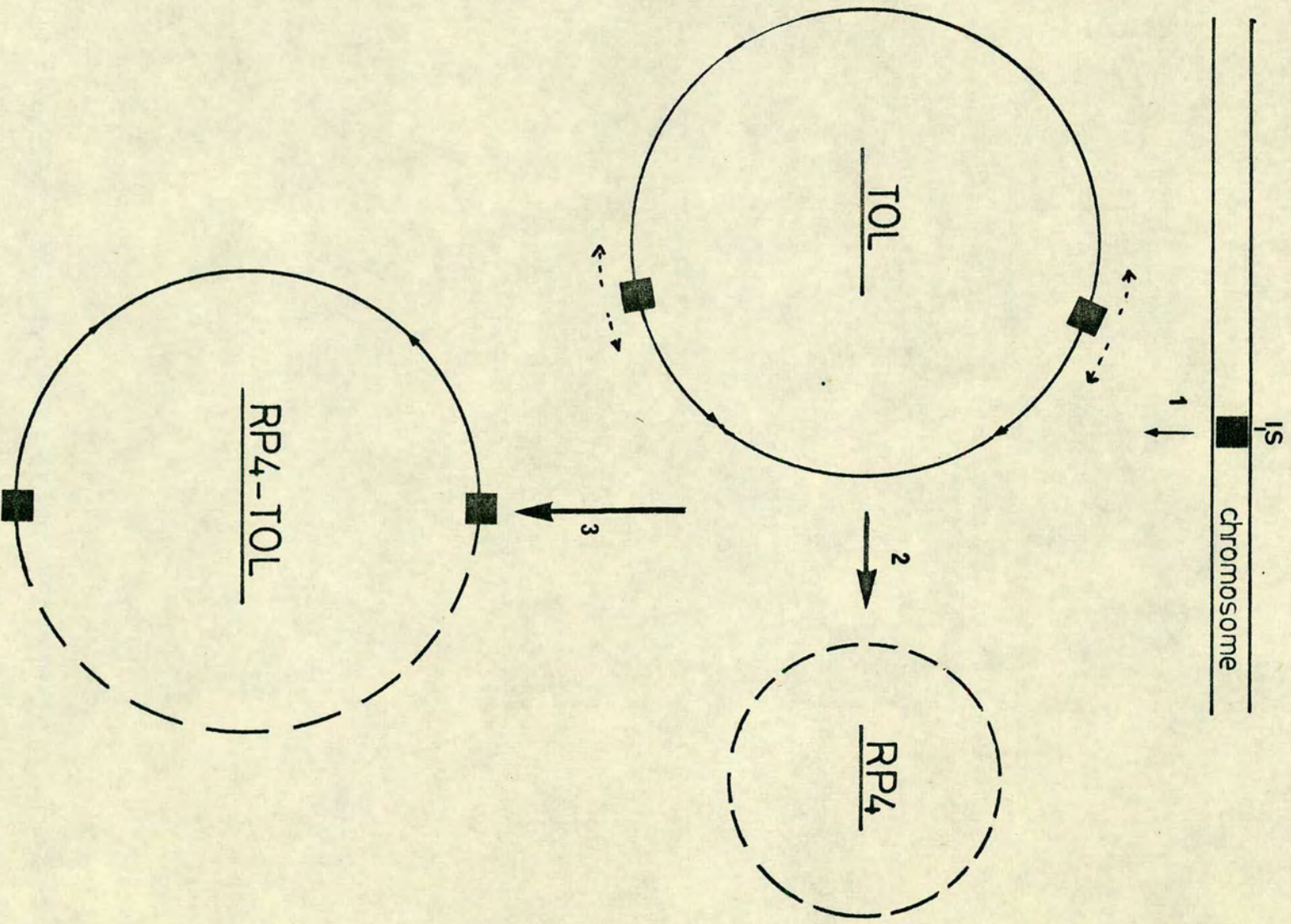
1. Insertion sequence (IS) from the Pseudomonas chromosome transposes to the TOL plasmid so as to span the tol genes (either simultaneous transposition or duplication of the element once on the TOL plasmid) which reside within the 40 kb excised region, the extent of which is indicated here by small arrows.
2. DNA between the two copies of the insertion element transposes to RP4 resulting in 3, the RP4-TOL hybrid.

This model explains why:

1. Variable segments of TOL DNA are found in the resulting hybrids.
2. Different insertion sites are possible on the recipient replicon.
3. Repeated sequence is found at each RP4/TOL junction.
4. A fragment containing the proposed repeated sequence shows homology with Pseudomonas chromosomal DNA.



Fig 56





these elements transposes onto the TOL plasmid so as to give two copies flanking the tol genes. The second stage in the process is thought to be the translocation of these genes into RP4. This model could explain why the TOL moieties found in the hybrid plasmids can vary in size. The Tol<sup>+</sup> phenotype is the selected character in the formation of RP4-TOL plasmids; that a segment of TOL DNA containing at least the 40 kb segment (which is thought to contain most or all of the tol genes) interacts with RP4 is therefore the only prerequisite for the formation of these hybrids.

There appear to be several preferential sites both on the TOL plasmid for insertion of the proposed insertion element, and on RP4 for the subsequent transposition of the tol genes. This is not an uncommon feature of transposable elements as preference for certain target sites for several such elements has been documented (Botstein and Kleckner, 1977; Grinsted et al, 1978). The most striking example of site specific insertion is that of IS4. Of the 20 IS4 insertions into the gal operon studied (Habermann et al, 1979), all were genetically inseparable and the three which were sequenced had insertions at identical nucleotide positions.

Since two RP4 TOL plasmids of Class A were made in P.putida (pED3300 and pED3301) and two in P.aeruginosa (pED3302 and pED3304), the proposed sequence involved in their formation must presumably be present in both species. This is supported by the observation that the PstI hybrid fragment from pED3305 showed homology to chromosomal DNA of both species. It is impossible to say at this stage whether the same sequence is involved in the formation of pTN2 or pED3303 as this was not tested due to the differences in TOL segments involved and their different insertion sites on RP4.



The insertion site of the TOL segment in pED3303 could not be finalised.

The studies of Jacoby et al (1978) showed that the TOL segment could be retranslocated from RP4 to pMG5. However, no molecular studies have been carried out on the pMG5-TOL recombinant to ensure that the identical segment was involved in both events. Moreover, these studies were not done in recombination deficient mutants of P.aeruginosa. The role of Pseudomonas insertion sequences in the events described in this Chapter therefore remain to be fully elucidated.



## CHAPTER 6. PLASMID-CHROMOSOME INTERACTIONS INVOLVING THE TOL PLASMID pWWO

### 6.1 Introduction

As stated in Chapter 3, strains derived from PAW1 which have lost the toluene degrading function can be selected for, after growth on benzoate, an intermediate in both the plasmid-encoded meta pathway and the chromosomally-encoded ortho pathway (Worsey and Williams, 1975). Such benzoate curing can occur either by loss of the plasmid from the cell, or by specific excision of a 40 kb DNA segment resulting in the formation of a plasmid conferring a Tol<sup>-</sup> phenotype, the first example of which was pWWO-8 (Bayley et al, 1977). The molecular basis for the latter event has been described in Chapter 3.

Morris and Broda (unpublished results) observed that DNA from a strain which had lost pWWO after benzoate selection (PAW86) shared homology with the TOL plasmid in DNA-DNA hybridisation studies. The possibility therefore arose that some plasmid-chromosome interaction was taking place similar to those occurring with the sex factor F (Jacob and Wollman, 1958) or the R factors R100 (Nishimura et al, 1973) and R68.45 (Haas and Holloway, 1976). In this study, three independently isolated plasmid-free strains derived from PAW1 by benzoate selection (Bayley et al, 1977) were analysed in order to assess the amount, if any, of pWWO DNA chromosomally located in each of these strains.

### 6.2 Some plasmid-free strains derived from PAW1 contain pWWO DNA sequences

Pseudomonas putida strains PAW82, PAW85 and PAW86 have previously been demonstrated to be plasmid-free on the basis that no closed



circular DNA molecules could be isolated from these strains by any of the methods normally used for the isolation of the TOL plasmid or its derivatives (Bayley et al, 1977). Total cellular DNA was isolated from these strains using the method of Dhease et al (1979), digested with endonuclease XhoI, run out on 0.7% agarose gels, transferred to nitrocellulose filters and hybridised to pWWO DNA,  $P^{32}$  labelled by nick-translation. DNA isolated from P.putida strain PAW1 (which carries the TOL plasmid pWWO) was used as a positive control and that from the plasmid-free strains A312 and AC34 as negative controls. Homology with TOL DNA was observed with the DNA from PAW85 and PAW86 but not with DNA from PAW82 (see Fig. 6.1). Such homology was confined to a few fragments which had total sizes of about 18 kb and 40 kb in the cases of PAW86 and PAW85 respectively. To test the hypothesis that such homology was due to the integration of a segment or segments of TOL DNA, it was necessary to establish the extent (as evidenced by the presence or absence of particular restriction fragments) of TOL DNA present chromosomally.

Fig. 6.1 shows the Southern blot of XhoI digested DNAs using TOL plasmid DNA as the probe. Inspection of these data, comparing the homology observed with the DNA from PAW85 and PAW86 to that of the pWWO containing strain (PAW1), indicated that the DNA on several XhoI fragments could not be present, in PAW85 and PAW86, in the form in which it exists in pWWO itself. These are: XF, XG, XC, XJ, XI, XE and XD, all of which constitute a continuous segment of the pWWO genome which includes HindIII fragment HA, and approximates to the 40 kb segment excised on benzoate selection (see Fig. 6.2). The presence or absence of the remainder of the



Fig. 6.1     Autoradiogram showing Southern blot of XhoI restricted Pseudomonas chromosomal DNA using pWWO as a labelled probe.     The numbered tracks contain DNA from P.putida strains 3. PAW1; 4. PAW82; 5. PAW85; 6. PAW86; 7. A312; 8. AC34; tracks 1 and 2 contain XhoI restricted plasmid DNA of pWWO and pWWO-8 respectively.



Fig 6.1

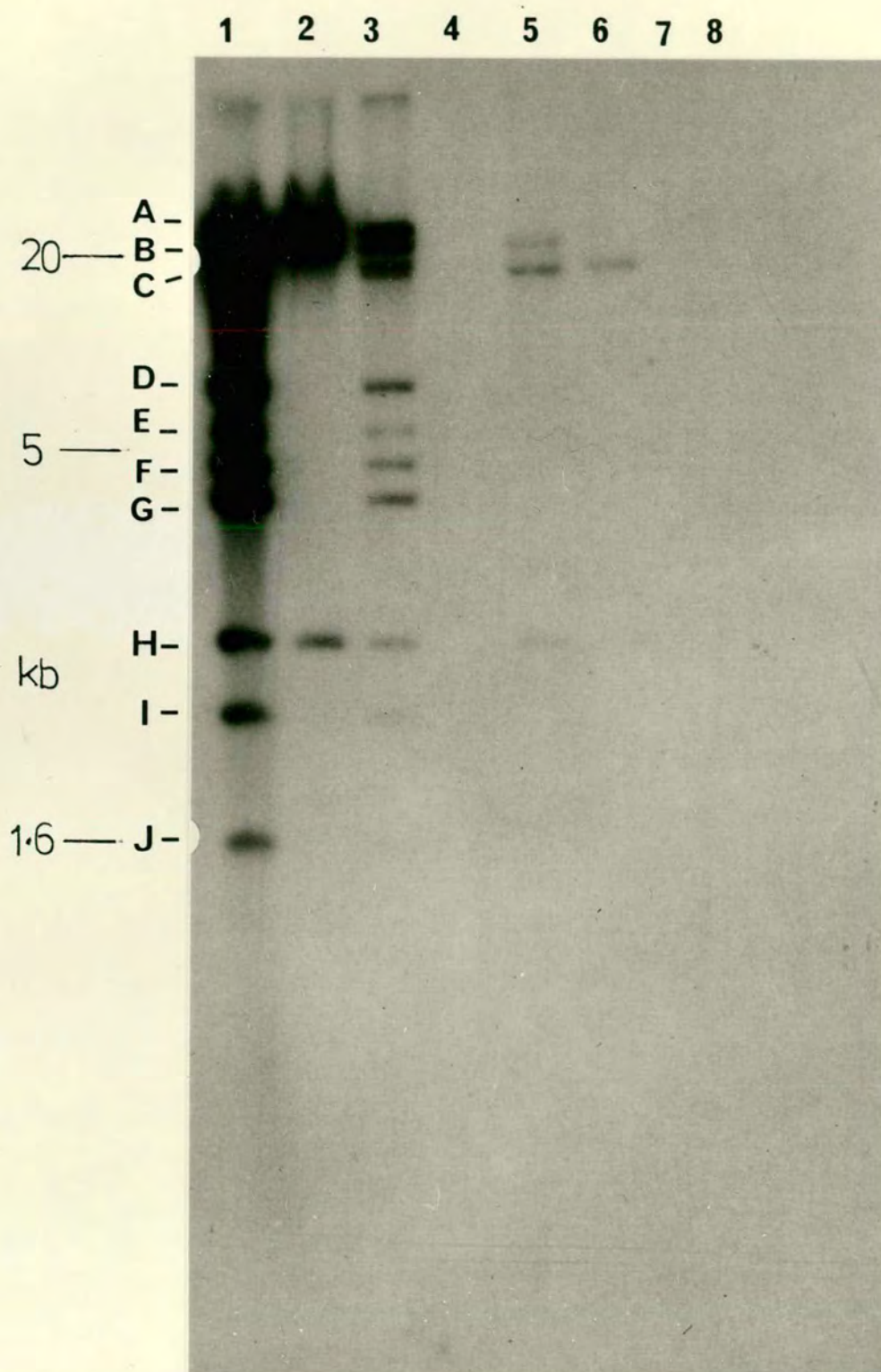
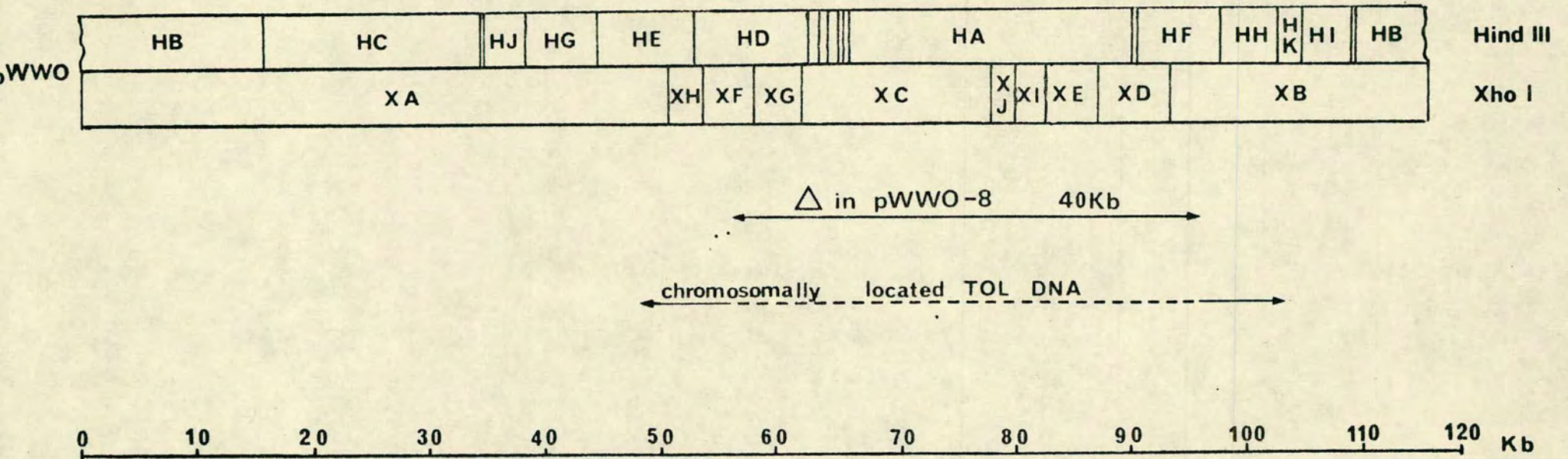




Fig6.2








Fig. 6.2 Physical map of pWWO. Cleavage sites are shown for XhoI and HindIII. The region deleted in the formation of pWWO-8 contains several XhoI cleavage fragments which do not appear to be present in the chromosomes of PAW85 or PAW86 both of which contain other TOL DNA sequences.

Also shown here is the region postulated as having a chromosomal location in PAW1. The dotted line indicates that the presence of the 40 kb segment in this strain is uncertain. The ends of the integrated segment are similar to those present in PAW85 and PAW86 (section 6.3).



pWWO genome was tested in hybridisation experiments using isolated HindIII generated fragments as specific probes.

### 6.3 The ends of the integrated TOL segment

DNA samples from PAW85 and PAW86 (that from PAW82 was sometimes used as a negative / <sup>control</sup> ) were digested with HindIII, run out on agarose gels, transferred to nitrocellulose filters and hybridised to specific HindIII fragments of TOL. These fragments, with the exception of HB and HH, had been obtained as clones in the vector plasmid pBR322 after a shotgun type cloning experiment with HindIII restricted pWWO DNA (R. Downing and P. Broda, unpublished results). The various probes were made by first digesting 2 µg of each recombinant plasmid with HindIII, separating the fragments on agarose gels, cutting out the gel slice containing the relevant fragment and extracting the DNA according to the method of Thuring *et al* (1975). This DNA was then <sup>32</sup>P labelled by nick-translation in the usual way. In the cases of HindIII fragments HB and HH, the fragments were obtained after HindIII digestion of pWWO DNA and subsequent separation on agarose gels. N.B. In order to resolve fragment HB, 0.5% agarose was used.

On hybridising the filters containing DNA from PAW86 with probes of HindIII fragments HH (5.0 kb), HE (8.0 kb) and HK (2.7 kb), fragments of 5.0, 6.3 and 1.6 kb showed homology respectively; conversely, negative results were obtained with HindIII fragments HB, HC, HJ, HG and HI (see Figs. 6.3 and 6.4). To explain these data it is suggested that a segment of the TOL plasmid, the ends of which are located in the HindIII fragments HK and HE, is integrated in the chromosome of PAW86.

When the same experiments were performed on DNA from PAW85,



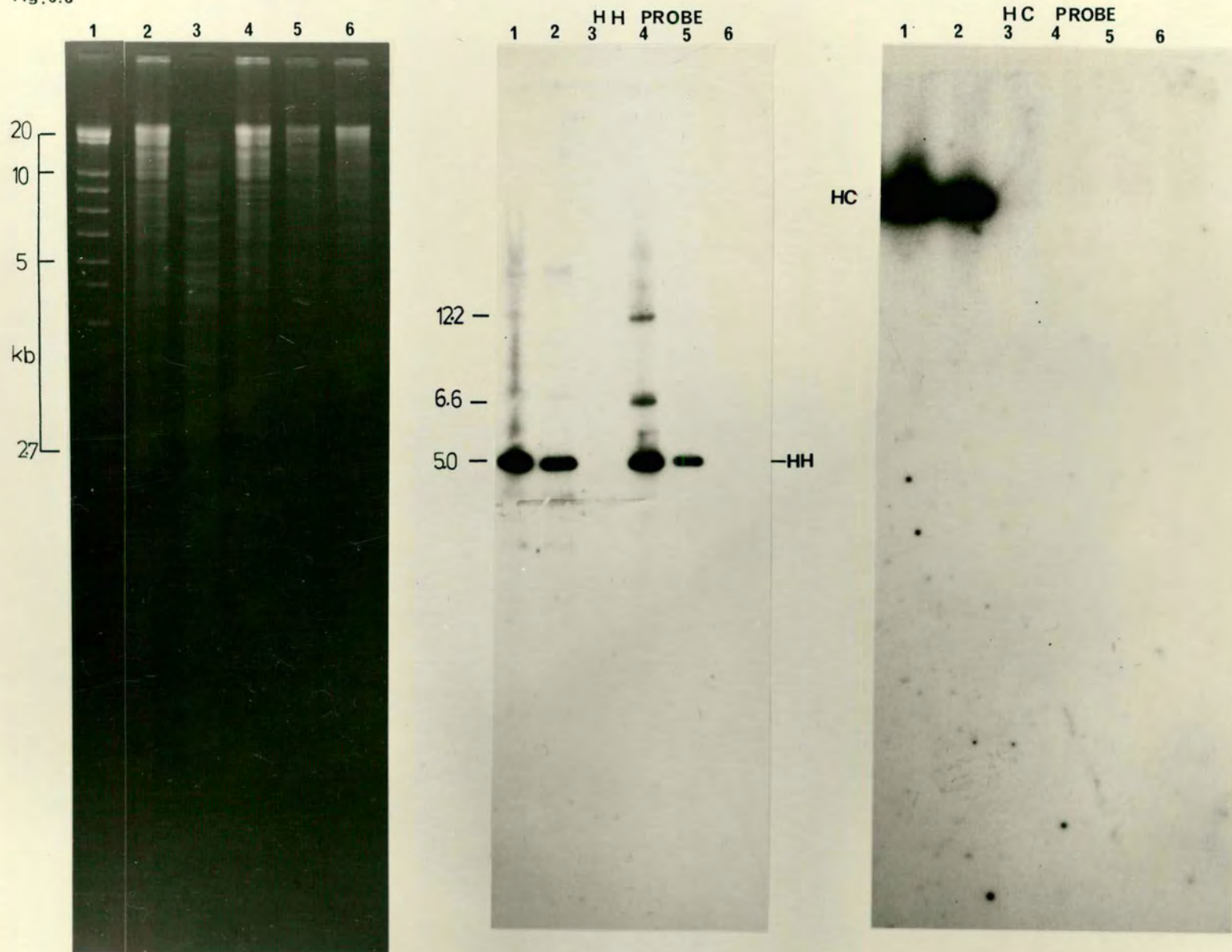
Fig. 6.3 Autoradiograms showing Southern blots of HindIII restricted chromosomal DNA hybridised to probes of HindIII restriction fragments HH and HC. Tracks 1-6 contain HindIII restricted plasmid DNA of 1. pWWO; and total cellular DNA from 2. PAW1; 3. PAW82; 4. PAW85; 5. PAW86; 6. AC34. When fragment HH was used as a probe, partial digestion products of 6.6 kb and 12.2 kb hybridised in track 4 indicating that the digestion of the DNA here was incomplete.

The main hybridising fragment is HH in the instances where homology is observed. This indicates that fragment HH must be intact in the chromosomes of PAW85 and PAW86.

Using fragment HC as the probe, not one of the tested strains showed homology whereas fragment HC present in the tracks containing the two positive controls (pWWO and PAW1) hybridised very strongly to the probe.



Fig. 6.3





HindIII fragments HK, HE and HH, all gave positive results. Negative results were obtained with the same fragments as in the experiment with PAW86. The interesting observation, however, was that fragments HK and HE each showed strong homology with two HindIII fragments of DNA from PAW85. In each case, one of the two fragments was identical in size to that hybridising in PAW86 with the appropriate probe (see Figs. 6.3 and 6.4). The same interpretation of the results obtained with PAW86 DNA is valid here except that two copies of the integrated segment appear to be present at different chromosomal locations.

#### 6.4 The parental strain, PAW1, also carries an integrated segment of TOL DNA

As stated above HindIII digests of total cellular DNA from strain PAW1 was routinely included as a positive control in the different hybridisations using the specific HindIII fragments as probes. In general, the expected results were obtained, namely that homology was observed only with the single fragment used as probe. However fragments HK and HE provided the exceptions as, in both cases, homology to one additional fragment of 1.6 kb and 6.3 kb respectively was observed. This indicates that in the parental strain, PAW1, there too seems to be a chromosomally located TOL segment.

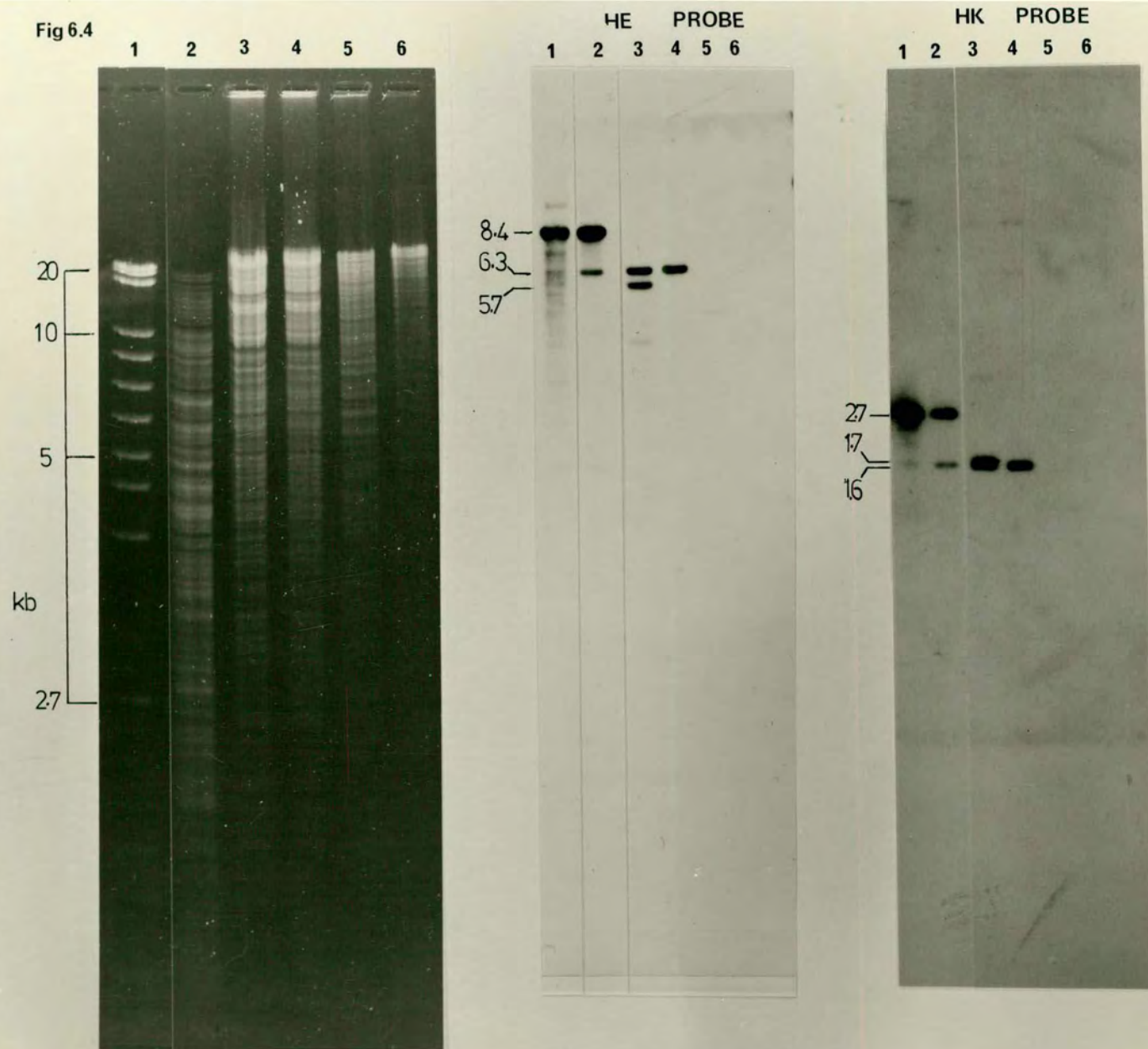
The evidence presented here would suggest that a segment of TOL plasmid DNA is present chromosomally. The segment stretches from HindIII fragments HK to HE on the intact TOL plasmid. In PAW85 and PAW86, the 40 kb excised region appears to be lost which would be predicted in these Tol<sup>-</sup> strains. Conversely, the segment in the PAW1 chromosome should contain all the DNA / <sup>between</sup> fragments HK



Fig. 6.4 Autoradiogram showing Southern blots of HindIII restricted chromosomal DNA hybridised to probes of HindIII restriction fragments HK and HE. HindIII digested pWVO DNA was used as a control (track 1). Tracks 2-6 contain total cellular DNA from strains 2. PAW1; 3. PAW85; 4. PAW86; 5. AC34; 6. A312. Whereas intact fragments HE and HK appear to be missing from the chromosomes of PAW85 and PAW86, some of the DNA present in these fragments is represented on differently sized chromosomal HindIII fragments in these strains. Fragments HK and HE hybridise each to one HindIII fragment in DNA from PAW86 (1.6 kb and 6.3 kb respectively) and to two fragments in the case of PAW85 (1.6, 1.7 kb and 5.7, 6.3 kb respectively).



Fig 6.4





to HE (56 kb). To test that these two proposals were correct, the presence or absence of the novel HindIII fragment Hd in pWWO-8 (indication of the loss of the 40 kb segment by precise excision) was tested for.

Although fragment Hd co-migrates with HindIII fragment HF from pWWO on agarose gels, whereas HF contains an XhoI cleavage site approximately 2.3 kb from one end, the XhoI site in Hd is only 0.2 kb from one end. These two fragments could therefore be distinguished in the following experiment.

Total cellular DNA from strains PAW1, PAW85, PAW86 and AC34 was digested with both XhoI and HindIII, run out on agarose gels, transferred to nitrocellulose and hybridised with  $^{32}\text{P}$  labelled purified fragment HF (the source of which was pED3307). The result, shown in Fig. 6.5, shows that the probe hybridises to a fragment of 7.2 kb to PAW85 and PAW86. Strong hybridisation to fragments of 2.2 kb and 4.7 kb, which correspond to the sizes of the XhoI digestion products of fragment HF, was evident in the track containing PAW1 DNA. However, hybridisation to a fragment of 7.2 kb was also visible in this track. This could be indicative of either incomplete digestion of the DNA (borne out in the track containing pWWO DNA) or of the integrated TOL segment in PAW1 having the same structure as those of PAW85 and PAW86. Further experiments are necessary in order to differentiate between these two possibilities.

#### 6.5 Stability of the TOL plasmid

If indeed there is a 56 kb TOL segment present chromosomally in PAW1 (which would stretch from fragment HK to HE on the pWWO cleavage map), then it should be possible to obtain a Tol<sup>+</sup>,



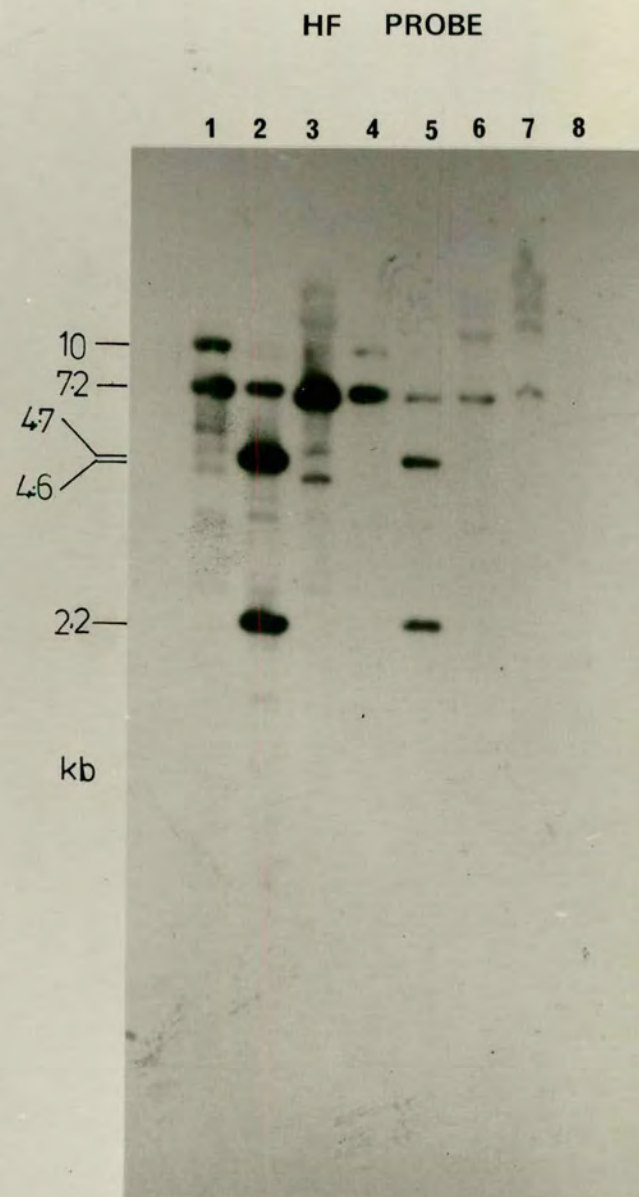
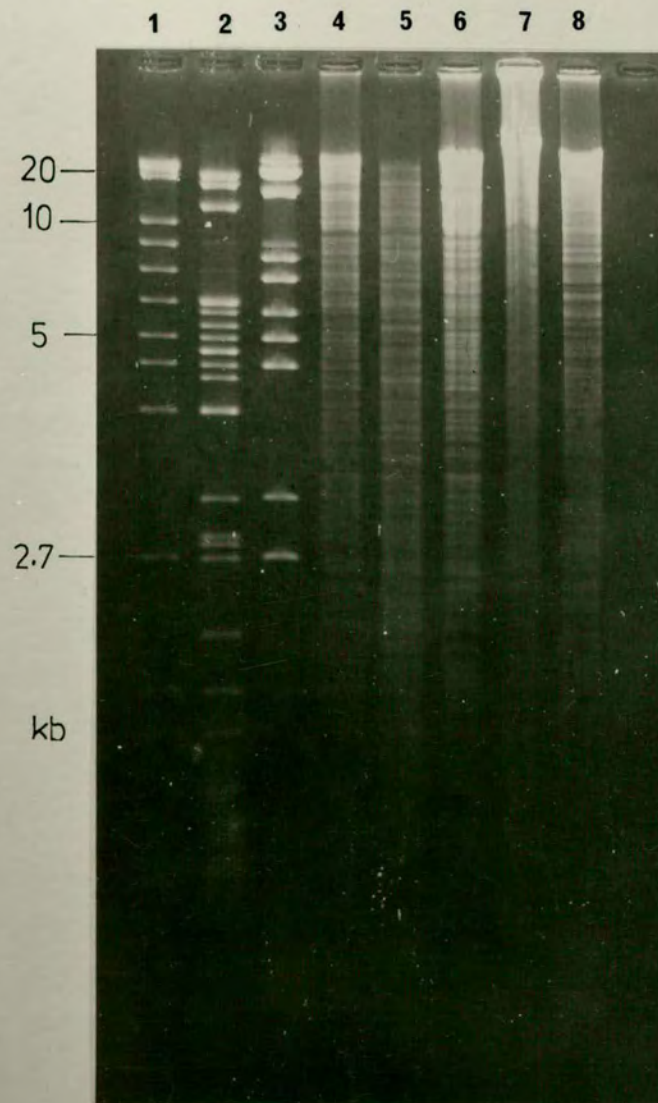
Fig. 6.5 Autoradiogram showing Southern blot of endonuclease digested chromosomal DNA hybridised to a probe of HindIII restriction fragment HF. Tracks 1-8 contain the following. 1. HindIII digested pWWO; 2. HindIII/XhoI digested pWWO; 3. HindIII/XhoI digest of pED3305; 4. HindIII of PAW1 DNA; HindIII/XhoI digests of total cellular DNA from 5. PAW1; 6. PAW85; 7. PAW86; 8. AC34.

Both fragments HD and HF hybridise to the probe in track 1 as these two fragments share a 1.4 kb of homologous DNA (Chapter 3). In the double digest (HindIII/XhoI) of pWWO the products of fragment HF are approximately 4.7 kb and 2.2 kb. Track 3 does not show these products as pED3305 has the novel fragment Hd produced on excision of the 40 kb segment from pWWO (section 4.3).

Hybridisation to a 7.2 kb fragment is observed in tracks 6 and 7.



Fig 6.5





plasmid free strain. A search for such a strain failed (see below). According to the hybridisation data (Fig. 6.4) if the TOL DNA present in PAW1 was either in the plasmid form or on the chromosome, the chances of isolating a strain which lacked pWWO but remained Tol<sup>+</sup> should have been high (1 in 10 to 1 in 100). This situation does not seem to arise frequently. After inoculating 50 ml L-broth with a single colony of PAW1 grown on nutrient agar, the culture was taken through several cycles of growth in L-broth; diluting the culture to ensure that a small number of cells (<100) were used in each successive inoculation. Cells were then plated out on nutrient agar and replica plated onto m-toluate plates. Not one of the 1,000 colonies tested in this way had lost the TOL function. Moreover, of 25 single colonies screened for the presence of plasmids, all contained plasmids identical (in HindIII restriction pattern) to pWWO (data not shown). This indicates a high level of stability of the TOL plasmid in PAW1.

To test whether a segment of the TOL plasmid could integrate into the chromosome of a genetically different strain of P.putida, pWWO was transferred to a streptomycin resistant derivative of A312 (EDPS100), a strain which had never been in contact with the TOL plasmid and whose DNA had been shown to share no common sequences with pWWO (Meulien and Broda, unpublished results). Tol<sup>-</sup> clones were obtained after benzoate selection in the usual way (section 3.1) and 25 independent isolates were screened for the presence of pWWO-8 type plasmids. Surprisingly, not one of the isolates contained a plasmid whereas the parental strain was shown to contain a plasmid identical to pWWO in the same experiment (data not shown).



When chromosomal DNA from ten of the Tol<sup>-</sup> isolates was hybridised to <sup>32</sup>P labelled HindIII fragments HE or HK, no homology was observed even after long exposure of the autoradiogram (data not shown). The benzoate curing of the TOL function in P.putida EDPS100 therefore seems to have different characteristics from that in P.putida PAW1.

## 6.6 Discussion

This study has shown that there is a segment of TOL DNA present in some plasmid free strains, derived from PAW1 by the benzoate selection procedure. This segment which is present in two copies in one isolate (PAW85) stretches from HindIII fragments HK to HE in pWWO-8 plasmid. The absence of the 40 kb segment in these strains has been demonstrated by the lack of certain XhoI fragments of pWWO and the positive identification of HindIII fragment Hd, the novel fragment found in pWWO-8 as a consequence of the excision event. It has also been demonstrated that PAW1 itself has a chromosomally integrated segment of TOL DNA. Although the ends of this segment seem to be similar to those present in PAW85 and PAW86, the exact nature of the DNA inbetween HindIII fragments HK and HE remains unresolved.

If the segment present in PAW1 was a contiguous piece of pWWO DNA from fragments HK to HE, it would be approximately 56 kb in size and as such would be similar to those segments of the TOL plasmid described by Nakazawa (1980; also this study) to be involved in the RP4-TOL plasmid pTN2, and by Jeenes and Williams (1981) as being implicated in the R2-TOL hybrids described by them. In that study, derivatives of Pseudomonas sp. B13 were described as containing a plasmid identical to pWWO-8, but which were



phenotypically Tol<sup>+</sup>. When the resistance plasmid R2 was transferred into this strain in order to expel the incompatible pWWO-8 plasmid, no change in the degradative phenotype was observed. Moreover, the toluate degradative function could be transferred, on a 56 kb segment of pWWO DNA, to another strain the segment being carried on R2. As the status of the 56 kb of TOL DNA in the donor strain was unclear it was proposed that it had a chromosomal location.

More evidence that a toluene degrading pathway could have a chromosomal location comes from the existence of a strain PAM1, which has the same genealogy as PAW1, is functionally Tol<sup>+</sup> but harbours a plasmid identical to pWWO-8. Again this plasmid could be eliminated without loss of the TOL function from the cell (Jeenes and Williams, 1981).

The fact that PAW82 carries no DNA homologous to the TOL plasmid could have arisen by the precise excision of the integrated segment. If this were the case, it would support the proposal of Jeenes and Williams (1981) that the fragments HK and HE define the ends of a translocatable DNA segment carrying the tol genes.



## CHAPTER 7. FINAL DISCUSSION

The TOL plasmid pWWO from Pseudomonas putida (arvilla) mt-2 displays a variety of structural rearrangements, three of which have formed the basis of this study. The major conclusions that can be drawn from the data presented in this thesis are as follows.

1. The excision of the 40 kb segment from the TOL plasmid pWWO occurs due to reciprocal recombination between two directly repeated sequences of 1.4 kb in size positioned at each end of the excised region.
2. This 40 kb segment is not implicated as such in the formation of RP4-TOL hybrids as, in all cases studied, the TOL moieties are larger than 40 kb and overlap the excised region at both ends. The variability in both the sizes of TOL segments involved and their insertion sites in RP4 has led to the proposal of a model to explain the mechanism by which such hybrids may be formed.
3. The TOL plasmid interacts with the chromosome of P.putida strain PAW1 as evidenced by the existence of chromosomally located TOL DNA sequences in PAW1 and strains derived from it.

Whereas the gross structural analysis of these events is presented here, the precise mechanisms by which these rearrangements occur remain unclear, in as much as it has not yet been possible to demonstrate: (i) whether the 1.4 kb repeated sequence present in pWWO is a translocatable element; (ii) the exact nature of the proposed insertion sequence promoting RP4-TOL plasmid formation; (iii) whether a region of the pWWO genome is active in a chromosomal interaction or whether the TOL DNA sequences chromosomally located are inherently part of the chromosomal material



of P.putida strain PAW1. The latter possibility is cast in doubt due to the existence of strain PAW82, derived from PAW1 which shared no homology whatsoever to the pWVO genome in the hybridisation experiments described.

It is reasonably likely that insertion sequences will be found to play a role in some if not all of these interactions. Similar structural rearrangements occurring in E.coli (section 1.2) are almost always associated with the presence of IS elements, which often serve merely as sources of homologous DNA for legitimate recombination events (Cullum and Broda, 1979). The primary function of transposable elements in these cases is to link non-homologous DNA sequences. An insertion element can link two such segments by flanking DNA sequences which can be subsequently mobilised, by transposition, to another replicon.

This is the mode by which RP4-TOL plasmid formation is envisaged. The model presented in Chapter 5 assumes the existence of active insertion elements on the Pseudomonas chromosome, a fact which is brought into contention by the data of Willetts et al (1981) in which the transposition of IS21 from R68.45 is discussed. Selection for R68 derivatives with chromosomal mobilising ability ( $Cma^+$ ) always resulted in the formation of R68.45 type plasmids. It was therefore suggested that insertion sequences are much rarer on the Pseudomonas aeruginosa PAO chromosome than on that of E.coli as this selection should also have resulted in R68::IS plasmids (R68 having picked up an insertion sequence from the chromosome). However another explanation could be that the formation of R68.45 plasmids occurs at a much higher frequency than the formation of an alternative structure and R68::IS plasmids would therefore rarely be seen.



There is considerable evidence for the presence of IS-like elements in the Pseudomonas chromosome. Franklin and Williams (1980) reported the isolation of an RP4-TOL plasmid in P.putida which had a mutation in xylA, the structural gene for xylene oxidase. This mutation renders the strain unable to grow on hydrocarbon substrates (toluene or xylene) but will still grow on alcohol or acidic substrates, e.g. meta-toluate. This mutation was subsequently shown to be due to an insertion of 3 kb of 'foreign' DNA into the HindIII fragment HD of pWWO (Meulien and Broda, unpublished results). A similarly sized sequence has been implicated in the xylR mutation of an R2::TOL plasmid isolated by Jeenes and Williams (1981) again in P.putida. Yet another mutation of a similar nature was described by Nakazawa et al (1980) as being responsible for the low level expression of the toluene degrading genes residing on an RP4-TOL hybrid plasmid pTN1 made in P.aeruginosa. All of these mutations were reversible due to the precise excision of the different 3 kb inserts and resulted in full expression of the tol genes. It remains to be seen whether these sequences are related and whether they display other properties of the insertion elements of E.coli.

It is interesting to speculate whether these elements are involved in other structural rearrangements occurring among degradative plasmids: the translocation of salicylate degrading genes (Chakrabarty, 1978), the fusion of the CAM and OCT plasmids (Chakrabarty, 1973) and the dissociation of the OCT (Chakrabarty, 1974) and NIC plasmids (NIC encodes the enzymes involved in the degradation of nicotine) (Thacker and Gunsalus, 1978). The results of the structural analysis of the TOL plasmid, some aspects of which have been dealt with in this thesis, should prompt the appropriate molecular studies on other degradative plasmids.



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