THE CONJUGATION SYSTEM AND INSERTION SEQUENCES OF THE IncN PLASMID R46

GEORGE M. COUPLAND

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for

my mother, father and brother.

A B S T R A C T

IncN plasmids commonly encode resistance to a number of antibiotics, and endow this property on potentially pathogenic bacteria. They are capable of transferring between bacterial cells by conjugation. The conjugation system of these plasmids is markedly different from the archetypal system of the F plasmid, and during the course of this work has been studied in detail for one IncN plasmid, R46.

The origin of transfer (oriT), the specific site from which the plasmid DNA is transferred has been examined using a previously isolated recombinant, pED939. The transposon Tn1725 was inserted in pED939 resulting in its insertion within the 900bp oriT$^+$ fragment. Two of these insertions showed reduced oriT activity. A series of deletions and sub-clones were constructed using restriction targets within Tn1725, and these led to the positioning of the nick site within 174bp, as well as to the identification of two other domains required for full oriT activity. These two domains still allowed optimum oriT activity when separated by 9kb of DNA.

Restriction mapping positioned the oriT containing fragment within R46, and showed it to be at the end of the DNA encoding the transfer proteins. Furthermore, a promoter was identified which transcribed away from oriT towards the transfer genes. The direction of transfer of R46 was determined and shown to be such that it transferred the transfer genes last.

Eleven IncN plasmids were tested to determine if they would efficiently mobilise R46 oriT. Seven did, but four did not. This suggested two different conjugation systems within IncN plasmids.

The nucleotide sequence of a 650bp region with full oriT activity was determined and the Tn1725 insertions positioned within it. Features of the sequence are discussed.

A series of transfer deficient point mutants of R46 were characterised, and 30 transfer deficient R46::Tn*1725* plasmids constructed. These should allow further characterisation of the proteins required for transfer.

Finally, R46 and the closely related IncN plasmid N3 were each shown to contain two copies of an insertion sequence which was called IS*46*. This was shown to be closely related to IS*15*.

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Abbreviations

Ap	ampicillin
Ant	antimony (III)
Asa	arsenate
Asi	arsenite
bp	base pair
Cm	chloramphenicol
kb	kilobase
Km	kanamycin
min	minute
Nal	nalidixic acid
Rep	replication
Res	restriction (*EcoRII*)
s	second
sfx	surface exclusion
Sm	streptomycin
Spc	spectinomycin
Sul	sulphonamide
Tc	tetracycline
Tp	trimethoprim
Tra	transfer

Throughout this thesis ug has been used in place of µg to denote microgram.

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CHAPTER 6 - CHARACTERISATION OF IS46 AN INSERTION SEQUENCE FOUND ON R46 AND N3.

CHAPTER ONE

INTRODUCTION

1(a) Plasmids

A plasmid is an extrachromosomal DNA molecule which encodes functions required for its own replication in its bacterial host cell. Plasmids are present in a wide range of Gram negative and Gram positive bacteria. Although most exist as covalently closed circles some are linear (Hirochika et al., 1984).

Plasmids have been classified according to their ability to co-exist in the same cell: two closely related plasmids will not co-exist and are said to be incompatible. This is an indication that their replication systems are similar, and can interact with one another. Generally plasmids of the same incompatibility group show extensive (>65%) DNA homology, whereas there is relatively little homology (<15%) among those of different groups (Grindley et al., 1973; Falkow et al., 1974). This has been demonstrated in heteroduplex studies, and liquid hybridisation experiments among IncFII (Sharp et al., 1973), IncW (Gorai et al., 1979), IncP (Villarroel et al., 1983) and IncN (Brown, 1981; Konarska-Kozlowska and Iyer, 1983) plasmids. This homology between closely related plasmids involves much more DNA than is required for replication, and includes, in particular, regions required to encode the conjugation (see later) functions of the plasmid. This explains the observation that plasmids of the same incompatibility group encode the same type of pili (extracellular structures required for conjugation).

The regions conserved between related plasmids have been termed the "backbone" of the plasmid molecule (Villarroel et al., 1983), and the differences are due to insertion and deletion of sequences often involving transposable elements. However, the IncP group does include three plasmids (R751, R906 and R772) which share little homology with each other, or with other IncP plasmids, and must represent separate lines of descent within the IncP group (Villarroel et al., 1983).

Plasmids were first identified as a consequence of their ability to transfer from one bacterial cell to another. This process is called conjugation. The F plasmid was recognised (Lederberg, 1952; Hayes, 1953) because it enabled strains of E.coli to transfer chromosomal genes. Similarly, antibiotic resistance plasmids could spread through a population of bacteria and make them resistant to a variety of antibiotics (see Watanabe, 1963). The conjugation systems of plasmids from different incompatibility groups are usually distinct. This was demonstrated in a number of ways, including: lack of DNA homology in regions which encode the transfer functions; different lengths of DNA required for conjugation (e.g. 32kb for IncF plasmids, but ca.20kb for IncN plasmids); and differences in the pili encoded by different plasmids (Bradley, 1980).

It is common for plasmids to code for resistance to one or more antibiotics. The IncN plasmid R46, for instance, carries genes determining resistance to ampicillin, tetracycline, streptomycin, sulphonamide and spectinomycin (Brown and Willetts, 1981). The observation that closely related plasmids share a common backbone, but carry genes determining resistance to different antibiotics led to the suggestion that these genes were inherited by most plasmids relatively recently, probably via transposition. This proposal of how plasmids may have evolved is supported by the discovery of plasmids from the same incompatibility groups as contemporary plasmids in strains stored before the use of antibiotics (Datta and Hughes, 1983). None of these plasmids carry genes which determine resistance to antibiotics, suggesting that these may have been inherited by plasmids in the last fifty years. Presumably these genes were already present in the population, but after the introduction of antibiotics were amplified via transposition.

1(b) This study of IncN plasmids

In the course of this work the IncN plasmid R46 was extensively studied. It was isolated from Salmonella typhimurium in a Brighton hospital (Datta and Kontomichalou, 1965), and was one of the first plasmids to be assigned to the IncN group. R46 is a conjugative plasmid, 51.7Kb in size, and encodes resistance to tetracycline, ampicillin, sulphonamide, spectinomycin and arsenate compounds (Brown and Willetts, 1981).

This thesis extends our knowledge of the conjugation system of IncN plasmids. Firstly, the origin of transfer (oriT, the site from which plasmid DNA is transferred, see later) of R46 has been studied in detail, and the nucleotide sequence of a 650bp region with full oriT activity has been determined. This work was extended to other IncN plasmids by measuring the efficiency with which they were capable of mobilising R46 oriT clones. Secondly, the transfer genes of R46 were examined by making a number of transfer deficient transposon insertions and point mutants of R46, and a method by which these mutants could be assigned to complementation groups is discussed.

Finally, R46, as well as two other IncN plasmids (N3 and pCU1), were shown to carry copies of the insertion sequence IS46.

2. IncN plasmids

2(a) Discovery of IncN plasmids

Plasmids were originally classified according to their ability to inhibit the fertility of the F plasmid (Watanabe et al., 1964), and Fi^- plasmids were initially all thought to be closely related and to produce I-type pili (Lawn et al., 1967). However it later became clear that some Fi^- plasmid-carrying strains would not propagate the I-type pilus specific phage If1. One such plasmid was R46, which was shown on the basis of incompatibility and surface exclusion to be closely related to N3 and R15, two of the classical Fi^- plasmids isolated by Watanabe. N3, R15 and R46 were placed in

incompatibility group N (Datta and Hedges, 1971), and their relatedness was emphasised by the discovery of a phage, IKe, specific for cells carrying an IncN plasmid (Khatoon and Iyer, 1971). Many more IncN plasmids have been described, and an extensive list has been compiled by Jacob et al. (1977).

IncN plasmids have a relatively wide host range. They were found to be present in species of Proteus, Providencia, Salmonella, Shigella, Klebsiella and Yersinia by characterising resistance plasmids transferred from these species to E.coli (Coetzee et al., 1972; Datta and Hedges, 1972; Hedges, 1974; Hedges, 1975; Hedges et al., 1973; Kimura et al., 1976; Jacob et al., 1977). Tardif and Grant (1980) reported that IncN plasmids from E.coli were transferred at high frequency to, and were stable in, the other genera of Enterobacteriaceae. Jacoby (1977) found that R46, but not N3 or R15, was transmissible to Ps.aeruginosa, suggesting that certain IncN plasmids can replicate in some Pseudomonas species.

2(b) Properties of IncN plasmids

The IncN plasmids R46, its derivative pKM101, pCU1 and N3 have been studied in some detail (Brown and Willetts, 1981; Langer et al., 1981; Langer and Walker, 1981; Konarska-Kozlowska and Iyer, 1981; Ando and Arai, 1981; Brown et al., 1984), and restriction endonuclease and genetic maps are available for each of these plasmids (fig 1.1).

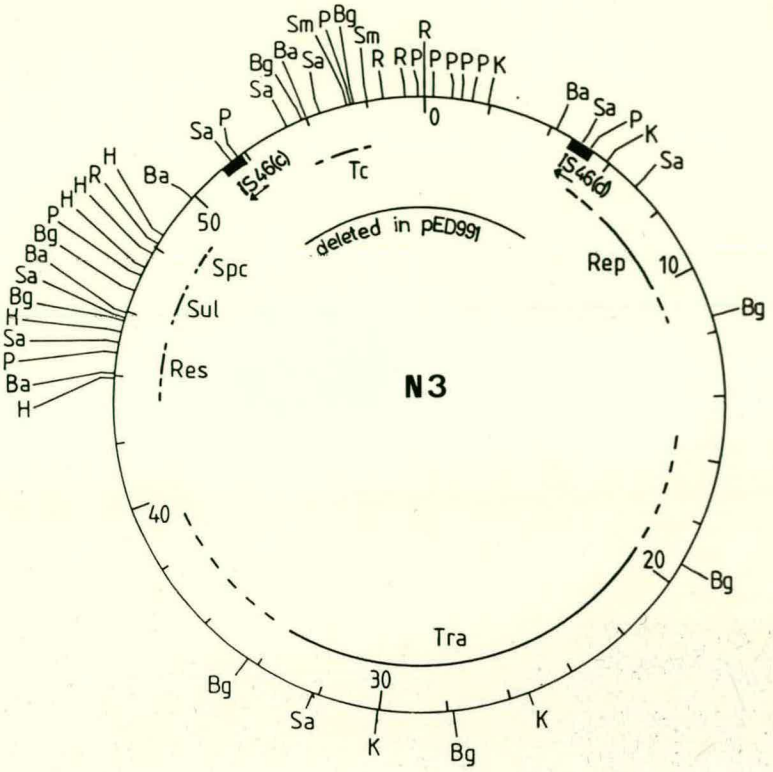
Brown (1981) made heteroduplexes between recombinants carrying the transfer region of R46 and the related plasmid N3. These suggested that the transfer regions of the two IncN plasmids were identical. Konarska-Kozlowska and Iyer (1983) analysed nine IncN plasmids (including pKM101) for homology with different regions of the previously characterised IncN plasmid pCU1. A 6kb fragment of pCU1 which encodes resistance to ampicillin as well as the maintenance functions of the plasmid was used as a probe in Southern blot analysis against the other plasmids. All shared homology with the

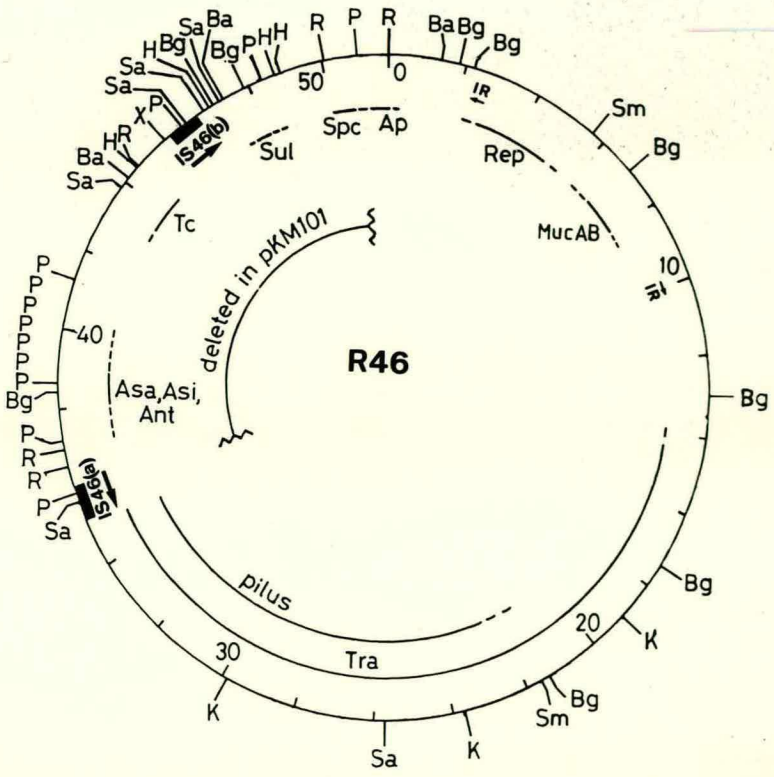
Fig. 1.1

Maps of R46 and N3.

The map of R46 was redrawn from Brown and Willetts (1981) with the addition of the two copies of IS46 (chapter 6; Brown et al., 1984). The restriction enzyme cleavage sites shown are EcoRI(R), HindIII(H), BglII(Bg), PstI(P), SalI(S), BamHI(Ba), KpnI(K), SmaI(Sm), and XhoI(X). Co-ordinates are in kilobases.

The map of N3 was redrawn from Brown (1981). The phenotype of N3 differs from that of R46 in that it does not carry Ap^R, Asa^R, Asi^R, or Ant^R determinants, its Tc^R gene is different, and it encodes the EcoRII restriction and modification system. The copies of IS46 are marked with heavy lines (see chapter 6; Brown et al., 1984).


N3
deleted in pED991
Tc
Spc
Sul
Res
Rep
Tra
IS46(c)
IS46(d)
0
10
20
30
40
50


R46
deleted in pKM101
Tc
Spc
Ap
Sul
Rep
MucAB
Asa,Asi,
Ant
pilus
Tra
IS46(b)
IS46(a)
IR
IRi
0
10
20
30
40
50

6kb fragment, suggesting that the maintenance functions are conserved between these IncN plasmids. Similar experiments showed that all the IncN plasmids shared homology with the transfer region of pCU1. It is therefore likely that, as with IncP plasmids, IncN plasmids have a common backbone consisting of maintenance and transfer functions.

IncN plasmids endow host cells with a variety of properties apart from antibiotic resistance. The characteristic properties of these plasmids are outlined below.

2(b)(i) Protection against effects of UV radiation

A number of plasmids, particularly of incompatibility groups I and N (Molina et al., 1979) enhance resistance of E.coli and S. typhimurium to uv radiation. Several such plasmids also increase the rate of mutagenesis induced by uv radiation or chemical agents, and increase the spontaneous mutation rate (Chernin and Mikoyan, 1981). The best studied of these plasmids is pKM101 which is an in vivo deletion derivative of R46 (Mortelmans and Stocker, 1979; Brown et al., 1984). These are the only plasmids which increase the propagation and mutation rate of uv-irradiated phage in unirradiated cells (Mortelmans and Stocker, 1976; Walker, 1977; Chernin and Mikoyan, 1981). The capacities of pKM101 to enhance survival after uv radiation, and to increase the rate of mutagenesis, are closely related as single mutations affect both processes (Walker, 1978). The ability of pKM101 to increase the rate of mutagenesis in the presence of a number of mutagens led to the inclusion of the plasmid in the Ames tester strains for the detection of potential carcinogens (McCann, 1975). The genes involved in this effect have been mapped in R46 and pKM101 (Brown and Willetts, 1981; Langer et al., 1981) and are called the muc genes.

In E.coli, R46 and pKM101 are able to suppress the effects of umuCD mutations (Walker and Dobson, 1979). The products of the umuC and umuD genes are thought to be involved in error-prone repair, as mutants in these genes are non-mutable with a wide range

of agents, but still exhibit the other SOS responses. The muc region of pKM101 contains two genes, mucA and mucB, with their products having the same molecular weights as the products of the umuD and umuC genes, respectively (Elledge and Walker, 1983; Perry and Walker, 1982). Furthermore, a gene fusion between mucB and lacZ expressed from the mucAB promoter, suggests that the mucAB genes are organised as an operon, and are, like umuCD, under the control of the lexA repressor (Elledge and Walker, 1983(b)). The genes are not induced in a strain containing the recA56 or lexA3 (ind^-) alleles. This is consistent with them being controlled in a similar fashion to other genes involved in the SOS response, which are induced in response to DNA damage by recA-mediated degradation of the lexA repressor.

Dowden and Strike (1982) have suggested that DNA present on R46, but absent in pKM101, codes for a repressor of the mucAB genes, and that this repressor may be interchangeable with the lexA protein. This may explain the observation of McCann et al.,(1975) that strains carrying pKM101 show a higher mutation rate than those carrying R46 itself.

2(b)(ii) Restriction and modification

Watanabe (1964) first showed that bacteriophage λ was restricted and modified by cells harbouring plasmids N3 and R15. This was extended by Bannister and Glover (1968) who showed that λ was restricted by only 12 of 151 strains carrying Fi^- resistance plasmids. These plasmids had the same specificity as R15 or N3, and this was called host specificity pattern II (hspII). It was later shown that all plasmids which carried the hspII system were IncN (Hedges, 1972), although not all IncN plasmids carried it. The genes required for restriction have been mapped on the N3 molecule (Brown et al., 1984). The sequence recognised by the hspII restriction system was determined by Bigger et al., (1973) to be $CC^{A}/_{T}GG$, and the modified bases were shown to be methylated cytosine residues within the restriction recognition sequence (Boyer et al., 1973). Smith and Nathans (1973) proposed a new nomenclature for restriction modification systems in which the hspII system was re-named EcoRII.

2(b)(iii) Potentially lethal products of pKM101

A number of plasmids have been shown to carry genes which are lethal to the host cell under certain conditions. The best understood of these are the lethal genes of RP4. There are three genes kilA, kilB and kilC which are lethal in the absence of the genes korA, korB and korC (for Kill over-ride; Figurski et al., 1982).

The IncN plasmid pKM101 encodes a similar system (Winans and Walker, submitted). It has been observed that cells harbouring pKM101 or R46 grow slowly on minimal medium, unless the agar is supplemented with purines (Langer et al., 1981), although why these plasmids should increase the requirement for purines is not clear. The region of pKM101 responsible for this phenotype was placed on the pKM101 map (Langer et al., 1981), and called slo. The product of the slo gene (now called kilA) is lethal in the absence of two other genes: korA and korB. A fourth gene kilB is also lethal in the absence of korA and korB, but in their presence may be necessary for pilus synthesis and conjugal transfer, as a Tn5 mutation of kilB is also transfer deficient. Alternatively, this could be explained by a polar effect of Tn5 on neighbouring genes.

Winans and Walker have shown that kilA and kilB cause cell death rather than loss of the plasmid. A plasmid which carries the korA and korB genes, and is temperature sensitive for replication will allow a cell also containing a plasmid with kilA and kilB, but no kor genes, to survive at 30°C. However, at 42°C the kor plasmid will not replicate and the cells die, even on medium without antibiotics.

Rodriguez and Iyer (1981) have reported killing of Klebsiella pneumoniae when it is used as a recipient in matings with strains carrying IncN plasmids. This effect appears to be specific for IncN plasmids, although IncP and IncW plasmids showed it to a lesser degree. It was dependent upon transfer, as all conjugation-deficient mutants were also deficient in killing (Kil^-). At low frequency Kil^-Tra^+ mutants could be recovered. The killing of K. pneumoniae appears to be unrelated to the kil/kor system of Winans and Walker as double mutants of kilA and kilB were still able to kill K.pneumoniae (Winans and Walker, submitted).

3. Conjugation

3(a) Introduction to conjugation

Conjugation is one method by which bacterial DNA can be transferred between bacterial cells. It requires cell to cell contact and is usually encoded by a plasmid present in the donor cell. There are two types of plasmid capable of transferring between bacterial cells: conjugative and non-conjugative plasmids. The former are generally large (>30kb) and encode all the products required for their own transfer. Non-conjugative plasmids, however, are usually small (<10kb), and although coding for some transfer proteins, they can only transfer when a suitable conjugative plasmid is present in the same cell to code for other transfer functions.

Conjugative plasmids have been isolated from a wide range of Gram negative bacteria (see Jacob et al., 1977), and are also present in Gram positive bacteria (Clewell, 1981). The following discussion will be concerned almost exclusively with the conjugative plasmids of E.coli, and particularly the F plasmid about which most is known.

3(b) Model for conjugation

3(b)(i) Cell-to-cell contact

Conjugative plasmids representative of all incompatibility groups have been shown to encode pili (Bradley, 1980). These extracellular structures appear to be required for conjugation (see below). There are three morphological types of pili as seen in the electron microscope: thin flexible; thick flexible; and rigid (Bradley, 1980). Where more than one naturally occurring plasmid from the same incompatibility group has been tested they produce the same type of pilus. The type of pilus produced can affect the efficiency with which the plasmid transfers in certain conditions. For instance IncN plasmids transfer at high levels on solid media, but approximately 1000 fold less efficiently when the matings are done in liquid media. This is thought to be due to their rigid pili being sheared off during mating in liquid media.

direction, the opposite strand of λ was transferred (Rupp and Ihler, 1968). The known polarity of λ DNA indicated that it was transferred with the 5' end leading. Vapnek and Rupp (1970) showed that only one strand of F plasmid DNA is transferred to the recipient, and that this is the denser in CsCl-poly (U,G) equilibrium density gradients, while the other strand remains in the donor cell. This supported the observation that F transfers single stranded DNA to mini-cells (Cohen *et al.*, 1968).

It is widely assumed that other plasmids transfer their DNA similarly, but there is little data available to support this. However, it has been shown that the IncFII plasmid R538-1 and the IncIα plasmid R64 *drd* 11 transfer only one strand to the recipient (Vapnek *et al.*, 1971). Further, RP4 (IncP) and R144 (IncIα) transfer their DNA unidirectionally (Al-doori *et al.*, 1982; Barth and Datta, 1976), but it is not yet proven that they transfer only one DNA strand.

Willetts (1972) realised that the nick introduced in one strand of the F plasmid must be specific, as the bacterial chromosome is not transferred. He concluded that there was a specific site on F at which the nick was introduced prior to transfer. This site was designated the origin of transfer (*oriT*). This hypothesis was confirmed by demonstrating that some Tra$^-$ Hfr strains which had suffered deletions within the F DNA were unable to transfer even if all the F *tra* functions were supplied *in trans*. Willetts concluded that these had lost *oriT*, and mapped this to one end of the transfer region (Willetts, 1972). Several conjugative and non-conjugative plasmids have since been proven to contain an *oriT* (reviewed by Willetts and Wilkins, 1984; see chapter 3).

Before a DNA strand can be transferred to the recipient a nick must be introduced at *oriT*. Everett and Willetts (1980) devised a system by which they could assay this nicking in a λ transducing phage which carries *oriT*. They showed that nicking requires two

F tra proteins: traY and traZ (chapter 5). The products of these genes are thought to form a specific endonuclease. Surprisingly, the transducing phage was nicked even in the absence of mating pair formation, and nicking must occur at oriT even in the absence of a recipient strain.

3(b)(iii) Triggering of transfer

Since the oriT of F is reversibly nicked during cell growth it must be transfer of the DNA that is initiated by mating pair formation. The model for F transfer (Everett and Willetts, 1980; 1982; Willetts and Wilkins, 1984) proposes that transfer of F DNA is triggered by an F encoded protein after mating pair formation. A candidate for this triggering protein is the traM product which is not required for pilus synthesis, formation of mating pairs, or nicking at oriT, but is essential for DNA transfer and the conjugal DNA synthesis which takes place in the donor to replace the transferred strand. Further, the location of the traM product in the inner membrane, and its ability to bind to DNA near oriT make it a potential candidate for a triggering protein (Achtman et al., 1979; Thompson and Taylor, 1982; Willetts and Wilkins, 1984).

3(b)(iv) Transfer of the DNA

Nicking at oriT and triggering of DNA transfer is followed by unwinding of the DNA to separate the single strands. An F plasmid protein, encoded by traI, is probably responsible for this unwinding as it has recently been shown to be DNA helicase I (M. Abdel-Monem et al., 1983). This protein possesses a DNA-dependent ATPase activity, and if it is fixed to the membrane it may supply the energy required for displacement of the transferred strand into the recipient. The DNA is unwound at an estimated rate of 1200bp per second, which is similar to the rate at which DNA is transferred (Abdel-Monem and Hoffman Berling, 1976; Kuhn et al., 1979; Willetts and Wilkins, 1984).

The role of the pilus in DNA transfer is not clear. The pilus may serve simply to recognise a recipient cell and bring the cells together, or in addition the DNA may be transferred through the hole in the pilus. Once mating aggregates have formed concentrations of SDS which would dissociate pili do not prevent DNA transfer, suggesting that either the pili are unnecessary for this or are protected at this stage (Achtman, 1978). Willetts and Wilkins (1984) pointed out that although the hole in the pilus is large enough to allow transfer of DNA, if specific proteins are transferred in association with the DNA, this may be incompatible with the complex being transferred through the pilus.

Further, there is evidence for fusion of the membranes of the donor and recipient cells during transfer. λ receptor protein can be exchanged between Hfr and F^- strains as a consequence of mating (R. Goldschmidt and R. Curtiss, quoted in Willetts and Wilkins, 1984). Moreover, some E.coli mutants which are deficient in their ability to act as recipients in matings with cells containing certain F-type plasmids have been shown not to produce the ompA major outer membrane protein. The ompA protein is transmembranous and may occur at the adhesion sites between inner and outer membranes (Di Renzio and Inouye, 1979) that have been implicated in nucleic acid transfer (Bayer, 1968). The pili on the donor cell also occur at these adhesion sites (Bayer, 1976). Perhaps retraction of the pili bring the adhesion sites of donor and recipient together, fusing the membranes, and allowing the plasmid access to the recipient cell via a specific membrane protein complex. This complex probably includes the protein encoded by traD of F, as this is present in the inner and outer membranes, and is involved in the penetration into the cell of certain plasmid specific phage. The traI protein may also be positioned on the membrane, near this complex, so that unwinding can supply the energy to move the DNA into the recipient.

3(b)(v) Circularisation of transferred DNA

F DNA enters the recipient as a linear single strand. Single stranded DNA is unstable in E.coli so it is likely that the transferred DNA is stabilised by single stranded DNA binding protein. Recently F plasmid has been shown to encode a single stranded DNA binding protein which shares extensive homology with that of E.coli (Klodkin et al., 1983). However deletion of the gene (ssf) which encodes this protein does not render the plasmid unable to transfer or unable to replicate, so its function is not clear. However, as the experiments used E.coli strains which were capable of producing single stranded DNA binding protein, it is possible that this compensates for loss of ssf.

The complementary strand to the transferred DNA is synthesised prior to circularisation. This was concluded for R1drd19 by finding linear double stranded plasmid DNA in association with the membrane of heavily irradiated recipients (Falkow et al., 1971), and is assumed to be the case for other conjugative plasmids.

Circularisation of plasmid DNA does not require expression of plasmid genes in the recipient. This was concluded from experiments in which F and ColIb-P9drd1 were replicated and circularised in rifampicin-treated recipients (Hiraga and Saitoh, 1975), and is inferred from the ability of oriT clones to circularise in cells which did not also inherit the conjugative plasmid.

The circularisation process is not understood in detail. A plasmid encoded protein bound to the leading (5') terminus of the DNA may recognise the other (3') end as it enters the recipient, and then allow circularisation. Whether there is any specificity required at the 3' end in the form of bound protein or nucleotide sequence is not known.

After circularisation the mating cells disaggregate, probably because of expression of transfer or surface exclusion genes in the recipient. The recipient cell, which now contains a double stranded, replicating plasmid is called a transconjugant and quickly develops the capacity to act as a donor.

3(b)(vi) Conjugal DNA synthesis

Vapnek and Rupp (1970) showed that the complement to the transferred strand of F plasmid was synthesised in the recipient, while in the donor DNA synthesis occurred to replace the transferred strand. This indicated that conjugation was a replicative process.

Both donor and recipient conjugal DNA synthesis (DCDS and RCDS respectively) of the F plasmid require DNA polymerase III (Wilkins and Hollom, 1974; Kingsman and Willetts, 1979). Early models proposed that the primer for DCDS was the 3' end of the transferred strand, by analogy with the rolling circle model for ØX174 replication (Gilbert and Dressler, 1968). However, this was disproven by showing that DCDS was inhibited by rifampicin, implying that it was necessary for RNA polymerase to synthesise a primer (Kingsman and Willetts, 1979). The primer for RCDS of F is synthesised by RNA polymerase and/or the primosome (Wilkins and Hollom, 1974; reviewed Willetts and Wilkins, 1984).

Several plasmids from different incompatibility groups, most notably IncI and IncP (but not IncN) encode a primase which synthesises an RNA primer on single stranded DNA templates *in vitro* (Lanka and Barth, 1981). Although the IncI primase is co-controlled with the *tra* genes, primase deficient mutants (*sog*$^-$) transfer at frequencies comparable to the wild type plasmid (Chatfield *et al.*, 1982). However, RCDS was virtually eliminated when a *sog*$^-$ plasmid was transferred to a *dnaG* primase deficient recipient. Interestingly, RCDS did occur if a non-transferable recombinant carrying the *sog* gene was present in the donor. This was almost certainly due to transfer of the primase with the plasmid DNA, and sets an important precedent for the transfer of transfer proteins from the donor to the recipient during conjugation.

CHAPTER TWO

MATERIALS AND METHODS

2(a) Growth Media and Buffers

L-Broth contained, per litre: Difco Bacto Tryptone, 10g; Difco Bacto yeast extract, 5g; NaCl, 5g. pH7.2.

Nutrient agar: Oxoid no. 2. Nutrient Broth, 25g; Davis New Zealand agar, 12.5g per litre.

L Agar: As L-broth, but with 10g NaCl and 15g Difco agar per litre.

BBL Agar: Baltimore Biological Laboratories trypticase, 10g; NaCl, 5g; Difco agar, 10g per litre.

LC Agar: Difco Bacto Tryptone, 10g; Difco Bacto yeast extract, 5g; NaCl, 5g; Difco agar, 10g; 0.4M $CaCl_2$, 4ml; 20% (W/V) glucose, 5ml; 0.25% (W/V) Thymidine, 4ml, per litre. pH7.2.

M9 Minimal Agar: Davis New Zealand agar, 20g; NH_4Cl, 1g; NaCl, 0.5g; Na_2HPO_4, 7g; KH_2PO_4, 3g; $MgSO_4\ 7H_2O$, 0.4g; Glucose, 2.5g; Thiamine, 2ug per litre.

Spizizen Bacto Agar: Difco Bacto agar, 16g; $(NH_4)_2SO_4$, 10g; K_2HPO_4, 70g; K_2PO_4, 30g; Sodium citrate, 5g. $MgSO_4$, 1g; Glucose, 2.5g; Thiamine, 2ug per litre.

Difco Top Agar: Difco Bacto agar, 7g; L-Broth, 40ml per litre.

BBL Top Agar: As BBL agar, but only 6.5g Difco agar per litre.

LC Top Agar: Difco Bacto Tryptone, 10g; Difco Bacto yeast extract, 5g; NaCl, 5g; Difco agar, 7g; 0.5M $CaCl_2$, 10ml per litre.

Citrate Buffer: Citric acid, 78g; Trisodium citrate dihydrate, 258g per litre.

Phage Buffer contained per litre: KH_2PO_4, 3g; Na_2HPO_4, 7g; NaCl, 5g; 0.1M $MgSO_4$, 10ml; 0.01M $CaCl_2$, 10ml; 1% (W/V) gelatin, 1ml.

TE Buffer: 10mM Tris-HCL (pH8.0), 1mM EDTA.

TES Buffer: TE containing 1% (W/V) NaCl.

TAE Buffer: 40mM Tris, 20mM Sodium acetate, 5mM EDTA (pH8.2).

TBE Buffer: 90mM Tris, 89mM Boric acid, 2.5mM EDTA.

SSC: 0.15M NaCl, 0.015M Sodium citrate.

2(b) Plasmids, Phages and Bacterial Strains

See tables 2.2, 2.3 and 2.4.

2(c) Conjugation Techniques

2(c)(i) Broth matings

These were used to construct strains containing F-type plasmids. Fresh 2mls overnight cultures of donor and recipient strains were used. 0.1ml of donor and 0.1ml of recipient were mixed in a test-tube with 0.4mls of L-broth. These were left to stand in a 37°C water bath. After 90 minutes a range of dilutions was made and 0.1ml spread on selective plates.

In broth matings of pED100, where no selection for the plasmid was available, the recipient strain was selected for, and the resulting colonies checked for their sensitivity to F-specific phages.

2(c)(ii) Quantitative filter matings

This method was used in quantitative matings of all IncN, IncP, IncW and IncM plasmids. Fresh 2ml overnight cultures of donor and recipient were diluted 1:20 in 10mls of L-broth and shaken in a 37°C water bath until they reached a density of 2×10^8 cells/ml. These cultures were then chilled on ice. 0.2mls of the donor was mixed with 1.8mls of the recipient in a chilled Universal bottle. The mixture was taken up in a 2ml syringe and passed through a 20mm diameter membrane filter (Millipore Corporation, pore size 0.45uM). The cells were retained on the filter, which was placed on a pre-warmed nutrient agar plate at 37°C for 30 minutes. The filter was then placed in a chilled Universal bottle containing 2mls of L-broth and vortexed for 30s to resuspend the cells. These were then diluted and plated on selective plates as required. The original donor culture was diluted 10^5fold and 0.1ml spread on nutrient agar plates, to determine the number of donor cells used. The plates were incubated overnight (nutrient agar) or for approximately 36 hours (minimal agar).
20 of the colonies from the donor culture were patched and replica

TABLE 2.1 FINAL CONCENTRATION OF ADDITIVES TO MINIMAL AND NUTRIENT MEDIA

Antibiotics	Minimal(ug/ml)	Nutrient(ug/ml)	L-agar(ug/ml)
Ampicillin (Penbritin)	50	40	40
Chloramphenicol (Chloromycetin)	50	50	-
Kanamycin (Kantrex)	25	20	-
Nalidixic acid	40	40	-
Spectinomycin	100	20	-
Streptomycin	400	400	-
Sulphonamide (Sulphadimidine)	100	-	-
Tetracycline (Achromycin)	10	20	-
Trimethoprim	50	-	-
Amino acids	20	-	-
Others			
Thymine	50	50	-
Tetrazolium	-	-	20
Galactose	-	-	8mg/ml

TABLE 2.2 PLASMIDS

Plasmid	Phenotype, Genotype or Description	Inc Group	Reference/Source
pBR322	Ap^R Tc^R		Bolivar *et al*. 1977
pBR322 ::IS*1*	Tc^R		M Chandler
pCU1	Ap^R Spc^R	IncN	Konarska-Kozlowska and Iyer, 1981
pED101	Ap^R, *galK* coding sequence		P. Mullineaux
pED822	Ap^R, F*oriT*$^+$ derivative of pED825		Everett and Willetts, 1982
pED825	Ap^R		Everett and Willetts, 1982
pED889	Sul^S Tra^- mutant of R46	IncN	Brown *et al*, 1984
pED904	*Hind*III - A fragment of R46, *in vitro* deletion	IncN	Brown and Willetts, 1981
pED935	$Tet^R Cm^R$ R46 Tra^+ derivative of pBR325		Brown and Willetts, 1981
pED938	Ap^R, R46 *oriT*$^+$ derivative of pED825		Brown, 1981
pED939	Ap^R, R46 *oriT*$^+$ derivative of pED938		Brown, 1981
pED957	R46::Tn*5*	IncN	Brown, 1981
pED967	$Ap^R Cm^R$, R46 *oriT*$^+$ derivative of pBR325		Brown, 1981
pED994	Kan^R, pML31::IS*46*		Brown *et al*, 1984

TABLE 2.2(a) PLASMIDS

Plasmid	Phenotype, Genotype or Description	Inc Group	Reference/Source
F	Tra^+	IncFI	Hayes, 1968
pHR9	promoter of *gal* operon in pK04		Newman *et al.*, 1982
pIP1091	pBR322::IS*15*		Labigne-Roussel and Courvalin, 1983
pKM101	$Ap^R Uvp^+ Tra^+ Slo^+$	IncN	Mortelmans and Stocker, 1979
pLG339	$Kan^R Tet^R$		Stocker *et al*, 1982
pME420	$Ap^R Cm^R Tet^S$ deletion of pBR325		J Watson
pML31	Kan^R, mini-F replicon		Timmis *et al.*, 1978
pMUR274::Tn*7*	$Tp^R Tra^+$	IncN	Datta and Hughes, 1983
pMUR545::Tn*9*	$Cm^R Tra^+$	IncN	Datta and Hughes, 1983
N3	$Sul^R Spc^R Tet^R$ *Eco*RII $Uvp^+ Tra^+$	IncN	Watanabe *et al.*, 1964
R1-19	$Ap^R Cm^R Km^R Sm^R Sul^R Tra^+$	IncFII	Meynell and Datta, 1967
R46	$Ap^R Sul^R Spc^R/Sm^R Tc^R Uvp^+ Asa^R Asi^R Tra^+$	IncN	Datta and Hedges 1971
R68	$Ap^R Km^R Tc^R Tra^+$	IncP	Chandler and Krishnapillai, 1974
R100	$Cm^R Hg^R Sm^R Sul^R Tc^R Tra^+$	IncFII	Egawa and Hirota, 1962
R269N	$Ap^R Sm^R Tc^R$ *Eco*RII Tra^+	IncN	Hedges, 1972
R388	$Sul^R Tp^R$	IncW	Datta and Hedges, 1972
R390	$Ap^R Spc^R Sul^R Tc^R$ *Eco*RII	IncN	Coetzee *et al.*, 1972
R446b	$Tc^R Sm^R$	IncM	Hedges *et al.*, 1973

TABLE 2.2(b) PLASMIDS

Plasmid	Phenotype, Genotype or Description	Inc Group	Reference/Source
R447b	Ap^R Kan^R Tra^+	IncM	Hedges *et al.*, 1973
R825	Ap^R Tra^+	IncN	Hedges, 1974
R893	Ap^R Sm^R Tra^+	IncN	Hedges *et al.*, 1973
R979	Ap^R Sm^R Kan^R Tra^+	IncN	Mathew and Hedges, 1976
RM98	Ap^R Sm^R Sul^R Tc^R Tra^+	IncN	Jacob *et al.*, 1977
RSF1010	Sul^R Sm^R	IncQ	Guerry *et al.*, 1974
Rtsl::Tn*1725*	Km^R Cm^R	IncT	R.Schmitt
pUB307	Km^R Tc^R Tra^+ Ap^S deletion of RP*1*	IncP	Bennett *et al.*, 1977
pSC101	Tc^R		Cohen and Chang, 1977

TABLE 2.3 BACTERIOPHAGES

Bacteriophages	Genotype	Reference or Source
EDλ4	b 515 b 519 c 1857 Sam7	Dempsey and Willetts, 1976
EDλ7	c 1857 Sam7	Willetts and McIntire, 1978
f1		Achtman et al., 1971
f2		Achtman et al., 1971
Ike		Khatoon et al, 1972
M13mp8		Messing and Viera, 1982
M13mp9		Messing and Viera, 1982
P1 vir a		Willetts, 1969
PR4		Bradley and Rutherford, 1975
VAλ3	EDλ4 exo::1S1	Willetts et al, 1981

TABLE 2.4 BACTERIAL STRAINS

Strain		Relevant Characteristics	Reference/Source
E.coli K-12	C-600	Leu^- Thi^- Thr^-	Appleyard, 1954
	X478	Leu^- Pro^- Pur^- Lys^- Sm^R	Curtiss and Renshaw, 1968
	CE60-103	Trp^- Lac::Tn*5*	D.Berg
	CE60-124	Trp^- Lac::Tn*5*	D.Berg
	ED24	Spc^R	Willetts and Finnegan, 1970
	ED395		Dempsey and Willetts, 1976
	ED3818	His^- Lys^- Trp^- Str^R Nal^R	Willetts and Maule, 1979
	ED3822	Rif^R derivative of JC3272	N. Willetts
	ED3886	$RecA^-$ Spc^R	Brown *et al.*, 1984
	ED8654	Met^-	Borck *et al.*, 1976
	HB101	Leu^- Pro^- Thi^- $RecA^-$	Boyer *et al*, 1969
	JC3272	His^- Lys^- Trp^- Str^R	Achtman *et al.*, 1971
	JC6310	His^- Trp^- $RecA^-$ Str^R	Willetts, 1975
	JE2571	Leu^- Thr^- Str^R	Ohtsubo, 1970
	JM101	Lac^- (F *lac pro traD*36 *lacZ* M15)	Messing *et al*, 1981
	RU2901	Thi^- Thr^- Leu^- Pro^- Nal^R	R. Schmitt
E.coli B			Studier, 1969

TABLE 2.4(a) BACTERIAL STRAINS

Strain	Relevant Characteristics	Reference/Source
Enterobacter aerogenes (ATCC13048)		Skerman *et al.*, 1980
Proteus morganii (ATCC25830)		Skerman *et al.*, 1980
Providencia stuartii 164		Smith *et al.*, 1976
Salmonella typhimurium LT2 (NCIB 10248)		Clowes and Hayes, 1968
Shigella sonnei		J. Govan
Pseudomonas aeruginosa PA02	Ser^-	Holloway, 1969

plated onto appropriate plates to ensure they carried the plasmids being tested. Similarly,if the donor contained more than one plasmid 20 transconjugants were tested to determine which had received both plasmids.

2(c)(iii) Quantitative plate matings

Donor and recipient cultures were grown overnight in L-broth. 0.1ml volumes of appropriate dilutions of donor cells and undiluted recipient culture were spread directly onto selective plates and incubated overnight. This method gave less efficient transfer of IncN plasmids than did filter matings, but was sufficient to distinguish between Tra^+ and Tra^- phenotypes. It was also used to construct strains containing IncN, IncP, IncM or IncW plasmids.

2(c)(iv) Replica plate matings

These were used as a preliminary screen to isolate Tra^- mutants. Donor colonies were patched onto nutrient plates and incubated at 37°C for approximately 8 hours or overnight. They were then replica-plated onto selective plates spread with 0.1ml of a fresh 2ml overnight culture of recipient. Patches were distinguished as Tra^+ or Tra^- after overnight incubation at 37°C.

2(c)(v) Isolation of transfer deficient point mutants

(v) (a) Screening for tra^- mutants after mutagenesis

ED395 containing R46 was grown overnight in 2mls of L-broth. This was diluted 1:20 in 10mls of L-broth, and grown to a cell density of 2×10^8/ml. The cells were pelleted in the bench centrifuge, washed in 5mls of 0.1M citrate buffer and again pelleted. The cells were resuspended in 4.5mls of citrate buffer and 0.5ml of 1mg/ml N-nitro-N-nitroso guanidine (NTG) was added. This mixture was vortexed and incubated for 30 minutes at 37°C. 20ul of the mixture was then added to 2ml of L-broth and shaken overnight.

The overnight culture was diluted 10^5-10^6 fold and plated on nutrient agar plates containing tetracycline. The resulting colonies were replica plate mated to selective plates spread with JC3272 and putative transfer deficient mutants purified. These were re-tested in replica plate matings, and finally in semi-quantitative plate matings.

(v)(b) Enriching for $PR4^R$ tra^- mutants

ED395 (R46), ED395 (pED1029) or ED395 (N3) were mutagenised with N-nitro-N-nitroso-guanidine as described above. 0.1ml of the resulting overnight cultures were spread on selective plates either undiluted or diluted 10-fold. 0.01ml of PR4 (titre $>10^{10}$ p.fu/ml) was spotted onto the lawn of cells. 10 colonies which grew within the area of phage lysis were streaked on selective plates on which 0.1ml of PR4 had been spread. 2ml overnight cultures were then made of single colonies and these were checked for sensitivity to PR4 in spot tests (section 2(d)(i)). Small scale plasmid DNA preparations were made on any which were $PR4^R$, and these were used to transform ED395. The resulting colonies were re-checked for sensitivity to PR4, and if again resistant were checked for residual transfer-frequency in plate matings to JC3272.

2(d) Bacteriophage techniques

2(d)(i) Ike and PR4 preparation and spot tests

0.1ml of ED395 (R46) was mixed with 0.1ml of phage lysate in 2ml of molten LC top agar and poured onto an LC nutrient plate. The multiplicity of infection was sufficient to give confluent lysis. After overnight incubation at 37°C top agar was removed and the plate surface flooded with 4ml of L-broth. The top agar was vortexed with this broth in a Universal bottle and the mixture centrifuged for 5 min (5,000g). The supernatant was then carefully decanted and contaminating bacterial cells lysed by treatment with Diethyl ether(for Ike)or removed by filtration (for PR4; pore size 0.45um, Millipore Corporation).

For spot tests, 0.1ml of static overnight culture was added to 0.25ml of molten LC top agar, and poured onto LC nutrient agar. 0.01ml spots of Ike and PR4 suspensions (approximately 10^6 pfu/ml in phage buffer) were then applied and left to dry. Sensitive strains were lysed after overnight incubation.

2(d)(ii) f1 and f2 preparation and spot tests

f1 and f2 were prepared as described above for Ike and PR4 except that ED395 (Flac) was used to propagate these phages.

2(d)(iii) λ lysates and isolation of λ DNA

To prepare λ plate lysates ca.10^6 p.f.u. were first adsorbed to 0.1ml of C600 (2 x 10^8/ml; grown in L-broth supplemented with 10mM $MgCl_2$) at room temperature for 10 minutes. This mixture was then added to 2.5mls of molten BBL top agar and poured over a fresh, wet L-plate. This was incubated overnight at 37°C. The top agar was removed and the plate surface flooded with 2ml of L-broth. The top agar was vortexed with this in a Universal bottle and the mixture centrifuged for 5 min (5000g). The supernatant was then carefully decanted and contaminating bacterial cells lysed by treatment with chloroform. The lysate was titred.

To prepare λ liquid lysates 500ml of L-broth (containing 100mM $MgSO_4$) in a 2l flask was inoculated with 25ml of overnight culture of C600. The culture was grown to an optical density of approximately 0.5 (at 650nm; ca. 2 x 10^8 cells/ml). Phage was added to a multiplicity of infection of 0.1-1.0. Cultures were shaken at 37°C and the optical density followed. When the optical density stopped falling (i.e. when all the sensitive cells were lysed) 0.5ml of chloroform was added. The cultures were shaken for 5-10 minutes and the cell debris was removed by centrifugation (16,000g, 10 min., 4°C). The supernatant was poured into 500ml flasks with 10g of polyethylene glycol (PEG) 6000 and 4g NaCl for each 100mls. These were dissolved by shaking and placed in the cold room overnight.

The PEG-phage precipitate was harvested by centrifugation (10 min, 16,000g), and resuspended in 5ml of phage buffer. The resuspended phage lysate was spun at 8000g for 5 minutes to pellet protein.

To further purify and concentrate the phage the supernatant was loaded onto a triple step gradient. The step gradient was made by dispensing 2ml of 1.7g/ml CsCl solution (9.36g CsCl in 7.64ml of phage buffer) into a Spinco 25.1 polyallomer tube. 2ml of CsCl solution (10.0g of CsCl in 15ml of phage buffer) was layered on top with a 5ml pipette. The third step was made by carefully layering 2ml of 1.3g/ml CsCl solution (9.75g of CsCl in 22.75ml of phage buffer) on top of the first two steps. The phage solution was layered on top of the three steps and the tube spun at 73000g for 180 min in the spin-out Spinco 25.1 rotor. The phage band was removed with a 5ml syringe in a volume of approximately 2ml. This solution was made up with phage buffer and CsCl to final volume of 12ml and density of 1.6g/ml.

To further purify the phage this solution was dispensed into a Beckman heat seal polyallomer tube, and centrifuged (90,000g, 40hrs, 15°C). The resulting phage band was removed with a 5ml syringe. The phage were then titred and, 5×10^{12} phage dialysed against 100 volumes of TE. The phage were transferred to an acid washed repelcoted, screw-capped tube and phenol extracted (phenol pre-equilibrated with TE). The extraction was repeated twice.

The phenol was removed by dialysis against 100 vols of TES; then three changes of TE over about 2 days at 4°C. The DNA concentration and protein contamination of the solution were estimated from measurements of OD_{260} and OD_{280} of a ten (or forty) -fold dilution, using a Zeiss spectrophotometer. Protein contamination was considered negligible if the 260/280 ratio was >1.7.

2(d)(iv) Pl transduction

0.1ml of Pl vir a (10^7pfu/ml) were mixed with 0.2ml of 'donor' cell culture (grown overnight in L-broth) and plated out on LC agar in LC top agar). The transducing lysate was harvested as for Ike, and titred. Transduction was achieved by mixing 0.1ml transducing phage lysate (10^8pfu/ml) with 0.1ml fresh overnight recipient culture and 0.1ml of 30mM $MgSO_4$, 15mM $CaCl_2$, incubating at 37°C for 20 min, and spreading on selective minimal agar (containing 1% (w/v) Na_3Citrate).

(e) Galactokinase assays

This method is based on that of Wilson and Hogness (1966). 25ml of L-broth was inoculated with 0.5ml of an overnight culture of the strain to be tested. This was shaken at 37°C to an O.D.650 of 0.2 - 0.25. 1ml of the culture was removed and dispensed into a 'snap-cap' polypropylene microfuge (Eppendorf) tube to which 40ul of lysis mix (100mM EDTA, 100mM DTT, 50mM Tris-HCL pH8.0) and 4 drops of toluene were added. This was mixed thoroughly by vortexing for 1 minute. These tubes containing lysed cells were shaken at 37°C for 30-45 minutes with the lids open to evaporate the toluene.

Reactions were carried out in Eppendorf tubes which contained 10ul of 'mix 1' (5mM DTT, 16mM NaF), 25ul of 'mix 2' (8mM $MgCl_2$, 3.2mM ATP, 200mM Tris-HCL pH7.9), 5ul of galactose solution (50ul of 2% galactose, 50ul of D-(1- ^{14}C) galactose (Amersham CFA 435), 900ul distilled water) and 10ul of lysed cells. This mixture was incubated at 32°C for 15 minutes. One tube was included which contained 10ul of distilled water instead of cells. After the incubation a 25ul aliquot from each tube was spotted onto DE81 paper (a 2.5cm diameter circle).

The samples on the DE81 paper were then washed in distilled water. One sample was not washed for use in the calculation (see later). Three washes at room temperature were used. The water was poured off and the paper dried at 95°C for 30 minutes.

The paper was then placed in scintillation vials containing 3ml of 0.4% Butyl PBD in toluene.

Galactokinase activity was determined from the calculation:

nM galactose phosphorylated/min/O.D.650 =

$$\frac{\text{(sample c.p.m. - blank (no cells) c.p.m)} \times 5000}{\text{time(min)} \times \text{unwashed (c.p.m.)} \times \text{OD650}}$$

(f) Plasmid DNA preparations

(f)(i) Small-scale cleared lysate preparations for screening purposes

1.4ml of stationary phase cultures were pelleted in Eppendorf tubes using an Eppendorf microfuge (30 sec). The cells were resuspended in 50ul lysis buffer (35mM Tris-HCL pH8.0; 70mM EDTA; 18% (w/v) sucrose; 1mg/ml lysozyme (Sigma)) and incubated at 37°C for 5 min. 50ul of Triton lysis solution were added (0.2% Triton X - 100 (v/v); 50mM Tris-HCL, pH8.0; 60mM EDTA), mixed, and the tubes left to stand at room temperature for 10 min. The lysates were cleared by centrifuging (microfuge, 5 min). 40ul of supernatants were mixed with 10ul cleared lysate loading buffer (SDS 5% (w/v); Ficol 10% (w/v); Bromophenol blue 0.1% (w/v), in TAE buffer), incubated at 65°C for 10 min and introduced into the wells of an agarose gel for electrophoresis. Approximate sizes of plasmids were estimated from the mobility of CCC forms on the gel, with reference to CCC molecules of known size.

(f)(ii) Small-scale plasmid DNA preparations

This method is based on that of Birnboim and Doly (1979). 1.4ml of a shaken overnight culture in an Eppendorf tube was centrifuged (microfuge, 30 sec) the pellet resuspended by vortexing in 100ul lysis solution (Tris-HCl 25mM, pH8.0; EDTA 10mM; glucose, 50mM; lysozyme (freshly added) 2mg/ml, and left on ice for 10 minutes. 200ul alkaline SDS denaturation solution (SDS 1% (w/v); NaOH, 0.2M) were added, mixed and left at room temperature for 5 min. When the mixture was clear 150ul sodium acetate (3M, pH5.0) were added and mixed by inversion. A white precipitate of RNA

and protein formed immediately. The mixture was left on ice for 30 min, and mixed by inversion several times. After centrifuging (5 min., microfuge) 400ul of supernatant were carefully removed to a clean microfuge tube, mixed with 1ml of ethanol (100%), and left at -70°C for 20 min. Centrifugation (microfuge, 5 min, 4°C) yielded a white pellet of DNA and RNA which was re-dissolved in 100ul of dilute sodium acetate (0.1M, pH6), and the addition of 200ul of ethanol. After a further 20 min at -70°C, and 5 min centrifugation at 4°C, the final pellet was dried in a vacuum desiccator and resuspended in TE. The plasmid DNA recovered by this method was sufficiently pure for restriction endonuclease digestion, and use in transformation.

(f)(iii) Large scale purification of plasmid DNA (PEG method)

This method is based on those of Clewell and Helinski (1970), and Humphreys et al. (1975). 1ml of fresh overnight culture was added to 1 litre of L-broth and grown by shaking overnight at 37°C. The cells were harvested by centrifugation (16,000g, 10 min, 4°C) and resuspended in 10ml of 25% (w/v) sucrose in 50mM Tris-HCL pH8.0. Maintaining the cells on ice, 1.5ml of lysozyme solution (20mg/ml in 250mM EDTA, pH8.0) were added, followed by 8ml of 250mM EDTA (pH8.0). After each addition the cell suspension was swirled intermittently on ice for 5 min. 12ml of Triton lysis solution (0.2% (v/v) Triton X-100; 50mM Tris-HCl; 62.5mM EDTA; pH8.0) were then added, thoroughly mixed by drawing up and expelling from a 25ml pipette, and the suspension left on ice (30 min) for lysis to occur. The lysate was cleared by centrifugation (27,000g, 45 min, 4°C) and decanted into a 50ml measuring cylinder (on ice). 3% (w/v) NaCl and 10% (w/v) PEG 6000 were added, dissolved by inversion, and the solution left overnight at 4°C. After pelleting by mild centrifugation (5000g, 2 min) the PEG-DNA precipitate was re-dissolved in 7ml TES buffer, and transferred to a 15ml glass tube (corex) containing 11gm of CsCl and 0.5ml ethidium bromide (Eth Br) solution (10mg/ml in water). Once dissolved, the solution was left on ice (15-30 minutes) before centrifugation (12,000g, 30 min, 4°C). Under

these conditions PEG was displaced from solution and formed a pellicle on the side of the tube. The supernatant was carefully decanted into a clean tube, and further 0.2ml EthBr (10mg/ml) added, and the density of the solution adjusted to 1.59-1.61g/ml.

Ultracentrifugation (90,000g, 40-60 hrs, 15°C) was carried out in Beckman heat-seal polyallomer tubes in a 50Ti rotor. The lower of the two flourescent bands (containing supercoiled plasmid DNA), viewed under long-wave uv, was removed through the side of the tube into a 2ml syringe using a hypodermic needle. The solution was then extracted 3 times with n-butanol (pre-equilibriated against saturated CsCl solution) to remove EthBr and dialysed extensively against TE buffer at 4°C.

Finally, DNA concentration and protein contamination of the solution were estimated as described for λ DNA.

(f)(iv) Large scale purification of plasmid DNA (phenol method)

The cells were grown, lysed and the lysates cleared exactly as described in section (f)(iii) except that 500ml of cells were used.

After the clearing spin the supernatant was poured into a 30ml glass tube (corex). An equal volume of phenol (pre-equilibrated against TE) was added and mixed with the supernatant by inversion. This mixture was then centrifuged (10 min, HB4 rotor, 10,500g, 4°C) and the aqueous layer removed with a 10ml pipette. Half of the aqueous layer was dispensed in each of two 30ml glass tubes (corex) and 1ml of 3M Na Acetate (pH7.5) added with two volumes of cold ethanol. These were mixed by inversion and placed at -70°C for 30 min. This was then centrifuged (15 min, HB4 rotor, 10,500g, 4°C) the ethanol poured off and the tubes left inverted for 10 min to remove the remaining ethanol. The pellets were resuspended in TE to a total volume of 12.5ml. RNAase(0.1ml, 1mg/ml), EthBr (0.5mg/ml) and CsCl (0.95g/ml) were added and the final volume adjusted to 17ml with a density of 1.55g/ml. This was then dispensed into polyallomer tubes and spun in the sorval TV-865B vertical rotor for 19 hours at 191,000g.

This technique is much faster than the PEG method.

(g) DNA techniques

All DNA solutions were stored at 4°C in TE buffer.

(g) (i) Ethanol precipitation

DNA was precipitated by the addition of 0.1 volume 3M sodium acetate (pH5.0) and 2 volumes of ethanol. This was usually carried out in an Eppendorf tube. The DNA was then precipitated at -70°C for 15 minutes followed by centrifugation (microfuge, 5 min, 4°C). Pellets were washed with ethanol (100% or 80%), left at -70°C for a further 5 min., centrifuged again for 2 min. and dried in a vacuum dessicator, prior to resuspension in TE buffer.

(g)(ii) Restriction endonuclease digestions

Appropriate quantities of DNA, restriction endonuclease, 10X restriction buffer and distilled H_2O were mixed thoroughly in an Eppendorf tube and incubated at 37°C(65°C for TaqI). 10X universal restriction buffer was 0.3M Tris-Acetate (pH7.9); 10mM magnesium acetate; 0.66M potassium acetate; 5mM dithiothreitol; 1mg/ml nuclease free Bovine serum albumin. Restriction endonucleases used were obtained from Boehringer Mannheim GmbH, Bethesda Research Laboratories Inc., New England Biolabs Inc., and NBL enzymes Ltd.

(g)(iii) In vitro recombination of DNA fragments

Ligations were carried out using T4 ligase in 1 x ligase cocktail (66mM Tris-HCL (pH7.5), 1mM EDTA, 10mM $MgCl_2$, 10mM DTT, 1mM ATP). 100ng - 2ug of cleaved DNA was diluted with water, or added directly to 10X ligation cocktail. The final volume was 20ul-100ul. T4 ligase was added and incubated overnight at 10°C.

(g)(iv) Transformation of E.coli with plasmid DNA

0.5ml of a fresh overnight culture was diluted to 20ml in L-broth and grown to a cell density of 2 x 10^8/ml by shaking at 37°C. When the cells had reached 2 x 10^8/ml they were poured into a

McCartney bottle and chilled on ice for 10 min. The cells were pelleted (5,000g, 5 min) and resuspended in 10ml of ice cold $MgCl_2$ (100mM) and immediately centrifuged again (5,000g, 5 min). The cells were resuspended in 10mls of cold $CaCl_2$ (100mM), left on ice for 30 min, pelleted again (5,000g, 5 min) and finally resuspended in 1ml of 100mM $CaCl_2$. The competent cells were used immediately for transformation.

Up to 100ng of DNA was added to 0.2ml of competent cells in a test-tube which was then kept on ice for 30 min. The test-tube was then placed in a 42°C water bath for 2 min. 0.5ml of L-broth was added, and the cells shaken at 37°C for 1-16 hours before plating on selective media for transformants.

(g)(v) Transfection of JM101 with M13mp8 and M13mp9

JM101 was always stored on minimal plates, which selected for the F prime plasmid essential for M13 infection. A single colony was used to inoculate a 2ml overnight culture, and the next day 1ml of this was diluted to 20ml in L-broth. The cells were grown to a cell density of 2 x 10^8/ml and made competent by a procedure similar to that described in g(iv) except that the $MgCl_2$ wash was omitted.

After the heat shock 2.5mls of BBL top agar containing 20ul of 24mg/ml XG (5-Bromo-4-chloro-3-indolyl-3-D-galactopyranoside in dimethyl formamide (DMF)), 10ul of 24mg/ml MTG (Methyl-B-D-thiogalactoside in DMF) and 0.05ml of an exponential culture of JM101 was added to the transfected cells. This mixture was vortexed briefly, poured on Spizizen bacto agar plates, and incubated overnight at 37°C.

M13mp8 and M13mp9 plaques appeared blue, whereas recombinant plaques were white.

(g)(vi) Nick translation

10uCi of (α-^{32}P) - labelled nucleoside triphosphate (dCTP, Amersham Radiochemicals) in ethanol solution was dispensed into an Eppendorf tube containing 5ul of distilled water and dried in a vacuum dessicator. 98ul of DNA polymerase I buffer (50mM Tris-HCL pH7.5 and 5mM $MgCl_2$), 2ul of nucleotide triphosphate mix (10ul of a 10mM solution of each of the nucleotide triphosphates added to 960ul of H_2O), 1ul DNAase(2 x 10^{-5}mg/ml), 1 unit of DNA polymerase I and 1ug DNA were added to the dried down labelled nucleoside triphosphate. This mixture was incubated at 10-15^{o}C for 2 hours.

100ul of phenol, 5ul of orange G, 30ul of calf thymus DNA (1mg/ml), and 65ul of TE were added after the incubation. This mixture was briefly vortexed and spun in a microfuge for 2 minutes to separate the layers. The aqueous layer was removed and passed through a 2ml G75 column to separate the labelled DNA from the unincorporated -^{32}P dCTP.

(g)(vii) DNA fragment purification

(g)(vii)(a) Agarose gels

After the DNA had been electrophoresed (section h(i)), the gel was stained with ethidium bromide (5ug/ml) to localise the required band. This fragment was then cut out of the gel using a scalpel. The slice of agarose was placed in dialysis tubing containing 1ml of TE. The tubing was then immersed in a shallow layer of TAE in an electrophoresis tank and the DNA electroeluted at 200V for 30 minutes. The polarity was reversed for 2 minutes to release the DNA from the wall of the dialysis tubing. The TE was then removed from the dialysis tube.

A small DEAE-cellulose (Whatman, DE52) column was constructed in a 200ul Gilson tip, and used to purify the DNA. The column was washed twice with 1ml of TE before the DNA containing sclution was

passed through it. The DNA was then eluted with 0.5ml of TE containing 2M NaCl. The eluate was extracted with phenol; then with ether, and ethanol precipitated. Fragments prepared in this way were suitable for ligation.

(g)(vii)(b) Acrylamide gels

The DNA was electrophoresed and stained to localise the required band as described in section h(ii). The fragment of gel containing the band was placed in a siliconised (pre-treated with a 2% solution of dimethyldichlorosilane in 1,1,1-trichloroethane) test-tube and 0.6ml of gel elution buffer was added (0.5M ammonium acetate, 70mM magnesium acetate, 0.1% SDS, 1mM EDTA). This was vigorously shaken overnight at 37°C. The 0.6ml of buffer was poured off and the tube washed with another 0.4ml of gel elution buffer. These samples were pooled and 2.5ml of n-butanol was added tc extract the ethidium bromide. This mixture was shaken and then centrifuged (6,000g, 5 min). The aqueous layer was removed and two equal amounts dispensed into two Eppendorf tubes. 750ul of cold ethanol was added to each tube, and the mixture placed at -20°C overnight to precipitate the DNA.

The next day the tubes were centrifuged (microfuge, 5 min), the pellet redissolved in 200ul of distilled water, 20ul of 3M sodium acetate added and then after the addition of 440ul of ethanol the samples were placed at -70°C for 30 minutes. The samples were centrifuged (5 minutes, microfuge) washed once in 80% ethanol and then dried down in the vacuum dessicator.

DNA prepared in this way was suitable for ligation.

(h) Gel Electrophoresis

(h)(i) Horizontal agarose gels

Agarose slab gels (15cm x 25cm; 0.7% - 2% agarose (w/v) in 200ml TAE buffer) were placed between two perspex tanks (each containing 500ml TAE buffer), and absorbant wicks('Access-matting ',

saturated with TAE buffer) were positioned at each end to make electrical contact between the gel and the buffer tanks. 30ul DNA samples were mixed with 20ul loading buffer (0.1% (W/v) bromophenol blue, 10% (W/v) Ficoll, in TAE buffer) and loaded by Gilson automatic pipette immediately after removal of the well-former. Each well was then filled to the top with TAE buffer and initial electrophoresis carried out at 150V for 15 min. After this period the wells were again filled with TAE, the gel covered with a thin plastic sheet ('Saran wrap'), and electrophoresis resumed at 45V for approximately 16 hours. The gel was then stained with ethidium bromide (5ul/ml) for 15 min, and de-stained in H_2O for 60 min. DNA was visualised by placing the gel on a long-wave uv transilluminator (Ultraviolet Products Inc., 365nm peak transmission). Photography was with Ilford FP4 film for an exposure time of 10s through a red filter (Haya R (25A)).

(h)(ii) Polyacrylamide gels

This method was based on that of Maniatis et al. (1975). 5% or 8% (w/v) acrylamide gels in TBE buffer were prepared by mixing appropirate volumes of 37.5% (w/v) acrylamide and 2% (w/v) bis-acrylamide stock solutions with 10X TBE and H_2O. The acrylamide to bis-acrylamide ratio was 29:1 (w/v) and the gel volume either 50ml or 80ml. When run overnight the gel also contained 5% (v/v) glycerol. 0.5ml of 10% (w/v) ammonium persulphate and 60ul TEMED (N,N,N',N' - tetramethylethylenediame, Serva) were added to initiate polymerisation. The mixture was poured between two vertical glass plates (either 20 x 20cm or 20 x 30cm) separated by 1.5mm perspex spacers at the side edges, and sealed at the bottom edge with water agar. A toothed perspex well-former was inserted at the top of the gel immediately after pouring, and 30-60 minutes allowed for polymerisation. The well-former and seal were then removed and the gel connected to a perspex gel apparatus containing 1 litre of TBE buffer in each tank. 20ul DNA samples were mixed with 5ul of loading buffer (50% (v/v) glycerol, 0.125% (w/v) xylene

cyanol FF, 0.125% (w/v) bromophenol blue, in TBE) and loaded with a Hamilton microsyringe. Large gels (20 x 30cm) were run overnight at 200V, small gels (20 x 20cm) were run for 3 hours at 310V. Gels were stained for 5 min. in ethidium bromide (5mg/ml), de-stained for 10 min in H_2O, and DNA bands visualised and photographed as in h(i).

(h)(iii) Sequencing gels

6% polyacrylamide gels were used. The buffer gradient gels were prepared exactly as described by Biggin et al. (1983). Gels were run at 40 watts (1200 volts) for 2-5 hours.

After running the plates were separated and the gel was fixed in 10% acetic acid, 10% methanol for 15 min. The gel, still on the glass plate, was left to stand on its end for 15 minutes to allow this solution to drain off. The gel was then transferred to Whatman 3MM paper, and dried on a Bio-rad gel drier. It was autoradiographed as described in section j(i).

(h)(iv) Estimation of DNA fragment sizes

The migration distances of standard fragments (of known molecular weight) were plotted against the logarithm of their sizes (using semi-log paper) and the sizes of unknown fragments on the same gel determined from the graph. The size standards were HindIII restriction fragments of EDλ7 (Sanger et al., 1982) various restriction products of pBR322 (Sutcliffe, 1978).

(i) Electron microscopy

Heteroduplexing and formamide spreading of DNA molecules were by the method of Davis et al. (1971). Molecules were mounted on parlodion grids, stained with uranyl acetate, shadowed with platinum and carbon, and photographed using a Siemans Elmiskop 101 electron microscope. Negatives were projected and the outlines of molecules traced and measured, using either a Keuffel and Esser

map measurer or a Ferranti Cetec Tablet and Digitiser. ØX174 replicative form (5.38kb) and M13 (6.23kb) molecules were included on the grids to provide double and single strand length standards respectively.

(j) Southern hybridisation techniques

(j)(i) Agarose gels

After running agarose gels were stained and photographed as described in h(i). The DNA was then denatured and transferred to nitrocellulose as described by Southern (1975). The filters were rinsed in 2 x SSC and baked at 80°C for 2 hours. The nitrocellulose filters were pre-soaked for 1 hour by shaking at 37°C in hybridisation fluid: 4 x SSC, 50% formamide, 0.1% sodium dodecyl sulphate, and 1X Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll). Hybridisation was carried out overnight at 37°C in a plastic bag containing hybridisation fluid (20ml for a 12 lane gel) plus 500ug of denatured salmon sperm DNA and 10^5 to 10^6cpm of denatured probe (labelled by nick translation, as described in section g(vi)).

After hybridisation, filters were washed twice for 1 hour under hybridisation conditions and twice for 1 hour in 2 x SSC. The filters were then dried at 37°C. Autoradiograms were usually exposed for 1 to 2 days at -70°C, using pre-flashed X-ray film (Dupont Cronex 4) and a phosphotungstate intensifying screen (Dupont Cronex).

(j)(ii) M13 plaque hybridisation

After cloning and transfection white plaques were picked onto a second plate which had been overlayed with 2ml of BBL top agar containing 0.1ml of an exponential culture of JM101. This was incubated overnight, and then placed at 4°C for 2 hours. A nitrocellulose filter was then laid on top of the agar for 1 minute. The filter was removed and placed plaques uppermost for 2 minutes

on blotting paper soaked in denaturation solution (1.5M NaCl, 0.5M NaOH). The filter was then placed in neutralisation solution (3M NaCl, 0.5M Tris-HClpH7.4) for 5 min, and finally washed in 2 x SSC. The filters were blotted dry, dried at 37°C for 20 min., and then baked at 80°C for 2 hours.

(k) Nucleotide sequencing

The R46 oriT region was sequenced using the M13 method of Sanger et al. (1977), Sanger et al. (1980), and Biggin et al. (1983). DNA fragments were cloned into M13 mp8 and M13 mp9 (Messing and Viera, 1982; gifts of C.K. Lister and D.J. Finnegan). The ligation mix was used to transfect JM101 as described in section 9(v).

Single stranded DNA (template preparations) were made from white plaques. JM101 was grown to an O.D.650 of 0.3 and 1ml aliquots dispensed in Bijoux bottles. White plaques were picked using toothpicks and resuspended in the JM101 culture. This mixture was shaken for 4-5 hours with the bottles on their side. The culture was then decanted into an Eppendorf tube and centrifuged (microfuge, 5 mins) at room temperature. The supernatant was transferred to another Eppendorf tube and 0.2ml of 2.5M NaCl, 20% PEG 6000 was added. This mixture was incubated at room temperature for 30 min. The M13-PEG precipitate was pelleted by centrifugation (microfuge, 5 min), and the supernatant drawn off in a pasteur pipette.

The tube was centrifuged again (microfuge, 1 min) and the remainder of the PEG solution removed. 100ul of TE and 50ul of phenol (pre-equilibrated against TE) were added to the pellet. The mixture was thoroughly mixed by vortexing, and left to stand at room temperature for 10 min. The layers were separated by centrifugation (2 min, microfuge), the aqueous layer was removed to another tube, and the DNA ethanol precipitated. After the first ethanol precipitation the DNA was resuspended in 100ul of TE and the precipitation repeated. The resulting pellet was washed in 1ml of

ethanol, and dried in a vacuum dessicator. The pellet was resuspended in 50ul of TE, and stored frozen at -20°C.

The reaction and annealing procedure was carried out as described by Biggin et al. (1983) for sequencing with ^{35}(S) α-thio-dATP (Amersham International, specific activity 400ci/mmol. The nucleotide reaction mixes were as follows:

'T': 0.125mMdCTP; 0.125mMdGTP; 0.00625mMdTTP; 0.25mMddATP
'C': 0.125mMdGTP; 0.125mMdTTP; 0.00625mMdCTP; 0.04mMddCTP
'G': 0.125mMdTTP; 0.125mMdCTP; 0.00625mMdGTP; 0.08mMddGTP
'A': 0.125mMdTTP; 0.125mMdCTP; 0.125mMdGTP; 0.05mMddATP

2uls of one of these reaction mixes was dispensed into each of four Eppendorf tubes with 2ul of primed template. The (^{35}S)-dATP was aqueous and was dispensed with the DNA polymerase I (large fragment), 2ul per tube (0.2 units/ul Klenow, 10mM DTT 10mM Tris-HCL (pH8.0); 0.5uCi (^{35}S) α-thio-dATP). 2ul volumes of these three solutions were dispensed onto the sides of the Eppendorf tube, and the reactions were initiated by centrifugation (10 sec., microfuge). After 20 minutes at room temperature 2ul of chase (all four dNTPs at 0.25mM) were added and the reactions left for a further 15 minutes. Reaction mixtures were then stored at 4°C or 2ul of dye mix (1mg xylene cyanol FF, 1mg bromophenol blue, 10mM EDTA in de-ionised formamide) was added before boiling. Samples were boiled for 3 minutes before loading onto a buffer gradient gel.

Autoradiography was as described in (i).

(1) Tn1725 mutagenesis

Construction of ED734 (JC3272::Tn1725)

The donor strain Rtsl::Tn1725/RU2901 was grown overnight in 2ml of L-broth at 30°C. Rtsl was transferred to JC3272 on nutrient plates containing Cm and Sm, and incubated overnight at 30°C. 50 of the resulting colonies were picked, patched onto nutrient plates and incubated at 30°C. The patches were then replica plated

onto nutrient plates containing Cm and Sm, and incubated at 42°C. Growth at this temperature eliminated Rts::Tn*1725* because the plasmid is temperature sensitive for replication. The resulting $Cm^R Sm^R$ patches were purified by streaking onto a CmSm plate and incubating again at 42°C. That the plasmid had been lost from these $Cm^R Sm^R$ strains was confirmed by showing that they had lost the Kan^R marker of the plasmid. One of these was ED734 and this was assumed to be an insertion of Tn*1725* into the chromosome of JC3272. This was supported by showing that R46 could transfer by conjugation Cm^R from this strain, and that this was due to insertion of Tn*1725* into R46 (chapter 5).

Mutagenesis of pED939 with Tn*1725*

This procedure is also based on elimination of Rtsl::Tn*1725* at high temperature. Rts::Tn*1725*/RU2901, grown at 30°C, was used as donor in a plate mating to pED939/JC6310 selecting $Cm^R Ap^R Sm^R$ transconjugants. These colonies were patched on nutrient plates, replica plated to Cm Ap Sm plates and $Cm^R Ap^R Sm^R$ growth was used to inoculate 2ml of L-broth which was shaken overnight, and the plasmid DNA isolated in a small scale plasmid preparation. This was used to transform ED8654, which was then plated on CmAp plates. To avoid identical siblings only one colony was picked from each transformation experiment. These transformants were shown to contain pED939::Tn*1725* plasmids (chapter 3).

CHAPTER THREE

THE LOCATION AND ACTIVITY OF THE R46 oriT

3(a) Introduction

As described in chapter 1, the F plasmid was shown to contain a specific site from which the DNA is transferred during conjugation, called oriT. This was concluded from the construction of dominant Tra$^-$ deletions of Hfr strains (Willetts, 1972). The oriT of F was cloned into a ColE1::Tn3 cloning vector (Achtman et al., 1978) and eventually sub-cloned into a pBR322 derived vector on a 385bp fragment (Everett and Willetts, 1982). As predicted these recombinant plasmids can transfer in the presence of a plasmid carrying all the F genes required for conjugation, and indeed this can be exploited as a cloning strategy.

A number of other conjugative plasmids have been shown to contain oriT sites using similar cloning strategies. Those plasmids from which oriT has been cloned include R1, R100 (N. Willetts, R. Everett and W. Smith, quoted in Willetts and Wilkins, 1984), ColIb - P9 (Wymbs and B. Wilkins, quoted in Willetts and Wilkins, 1984), RK2 (Yakobson and Guiney, 1983), R46 (Brown, 1981), and R6K (Shafferman and Helinski, 1983). The nucleotide sequences of DNA fragments carrying the oriT of several of these plasmids are available, and are discussed in chapter 4.

Non-conjugative plasmids also have an oriT site. Those from ColE1, RSF1010 and CloDF13 have been studied in detail (Warren et al., 1978; Derbyshire, 1983; Snijders et al.,1983). Mobilisation of recombinants containing the oriT of these plasmids requires proteins encoded by a suitable conjugative plasmid, as well as other proteins determined by the non-conjugative plasmid itself.

In all plasmids studied in detail the oriT sequence maps to one end of the region which encodes the proteins involved in conjugation. This was first shown for F (Willetts, 1972), where oriT maps near traJ (see Willetts and Skurray, 1980). In plasmids RK2 and R46 where there are three or more tra regions the oriT sequence has been mapped to one end of one set of tra genes (Guiney and Helinski, 1979; Winans and Walker, submitted; this chapter). Even in the non-conjugative plasmids ColE1 and RSF1010 the oriT sequence is at one end of the DNA which encodes the mobilisation proteins (Warren and Sherratt, 1978; Derbyshire, 1983). Furthermore, in the two cases where the direction of transfer is known, RK2 and F, the DNA is transferred such that the tra region is transferred last.

Specificity of oriT: The oriT sequence and the gene products which interact with it are generally plasmid specific. Many F-like plasmids (those from Inc groups FI, FII, FIII and FIV) have transfer operons which are very similar to F and will complement many F tra point mutants (Alfaro and Willetts, 1972). However, Reeves and Willetts (1974) showed that R100-1 and R1-19, both of which complemented 9 of the 11 Ftra^- point mutants tested by Alfaro and Willetts, would not promote transfer from F oriT. This suggested that although the majority of their tra products are very similar to those of F, the ones required for the recognition of oriT, and presumably the oriT sequence itself, are different. On the other hand ColV2 (IncFI) and ColVB trp (IncFIV) both of which promoted transfer from F oriT (Reeves and Willetts, 1974), and complemented all the point mutants tested by Alfaro and Willetts (1972) probably have an identical transfer system to that of F. Everett and Willetts (1980) showed that R100-1 and R1-19 (encoded proteins) would not nick an F $oriT^{\lambda}$ transducing phage. This agreed with the observation that traY mutants (unable to form the traYZ endonuclease) of F are not complemented by R100-1 or R1-19 (McIntire and Willetts, 1980; Thompson and Achtman, 1979).

F traZ mutants are complemented by R1-19, but not R100-1 (Everett and Willetts, 1980). These data suggested that very closely related plasmids have different oriT sequences, and different gene products which recognise them.

Similar data have been reported for IncP plasmids (Yakobson and Guiney, 1983(b)). The RK2 (IncP) oriT clone was used as a probe against various IncP plasmids in Southern blot experiments and showed that the intensity of hybridisation, and size of the oriT containing fragment varied for certain IncP plasmids. There are two groups of IncP plasmids according to these criteria: a group which after digestion with HaeII gave strong hybridisation to a fragment of the same size as the HaeII fragment containing RK2 oriT; and a second group which gave weaker hybridisation to a smaller fragment. The data correlated with the genetic experiments as the second group gave low level mobilisation of the RK2 oriT clone while the first group gave high level mobilisation. The variation in the strength of homology suggested that closely related IncP plasmids encode different oriT sequences and different proteins required for their recognition.

R46 oriT: A. Brown (1981) commenced characterisation of the oriT of R46. He showed that deletions of R46 which had lost the BglII-F fragment could not transfer, even if all of the transfer genes were supplied in trans. Recently, using further deletions of pKM101, Winans and Walker (submitted) showed that a 1.2kb region within this BglII-F fragment contained oriT.

Sub-clones of R46 which contain oriT have also been constructed. A. Brown cloned the 4.6kb BglII-F fragment into the BamH1 target of pBR322, and showed that this plasmid could be mobilised at high frequency by derivatives of R46. By selecting for the ability to be mobilised by R46 derivatives R46 oriT was sub-cloned on a 1400bp fragment (in a plasmid called pED938, fig.3.1), and ultimately on a 900bp HaeII-TaqI fragment (in pED939) which retained full oriT activity. Sau3A, MspI and AluI restriction maps of the latter

fragment were determined. Finally a 215bp Sau3A fragment was sub-cloned, yielding a plasmid which was mobilisable, but at a much lower frequency than pED939 (Brown, 1981).

This chapter describes the further characterisation of the sequences required for R46 oriT activity. These experiments have led to a more detailed understanding of the sequences involved in the recognition of oriT, and to a more accurate positioning of the nick site itself. Moreover, the study has been extended to other IncN plasmids by testing their ability to mobilise R46 oriT clones. This has revealed specificity of oriT, and of tra proteins, among closely related IncN plasmids. The possibility that R46 oriT activity is activated by transcription across oriT was examined by fusing fragments to a galK gene in a promoter cloning vector. No promoters transcribing towards oriT were found, but one was shown to transcribe away from oriT. As with oriT clones from RSF1010, ColE1 and F, dimers of an R46 oriT clone were shown to monomerise during mobilisation. This is evidence for breakage and re-union at the R46 oriT during mobilisation. Restriction analysis of R46 oriT clones allowed the co-ordinates on the R46 map of the 900bp TaqI-HaeII fragment containing oriT in pED939 (hereafter referred to as the 900bp oriT fragment) to be determined. Finally, evidence for the direction of transfer of an R46 oriT clone, and thus of R46, is presented.

3(b) Restriction map and transposon mutagenesis of pED939

Using the restriction targets for MspI, AluI and Sau3A mapped by Brown, it had not proven possible to derive sub-clones of pED939 with complete oriT activity. Attempts were made to overcome this difficulty by extending the available restriction data. A restriction map of pED938 for the enzymes HinfI and HaeIII was made: the relevant portion is shown in fig 3.1. However, the targets for these enzymes did not help and the shortage of suitable restriction targets within the 900bp oriT fragment made it necessary to sub-clone oriT from pED939 without relying on restriction targets

already available in the plasmid. To this end it was decided to make transposon insertions into pED939.

The Cm^R, Tn3-like transposon Tn1725 was used (Altenbuchner, et al., 1983). This is flanked by inverted repeats, which contain EcoRI targets 15bp from each end of the element (Schöffl et al., 1981). The method used to insert Tn1725 into pED939 is described in chapter 2.

Small scale plasmid preparations were made from the Cm^R Ap^R (putative pED939::Tn1725 transformants. Cleavage with EcoRI gave three fragments: the 8.9kb internal fragment of Tn1725, and two others. The combined size of the latter was equal to the size of pED939 (2.9kb). This confirmed that the plasmids arose from insertions of Tn1725 into pED939, the sizes of the two smaller fragments representing the distances from the single EcoRI target of pED939 to those at the ends of Tn1725.

Only those plasmids which yielded an EcoRI fragment of less than 900bp could have Tn1725 inserted within the 900bp oriT fragment; these were studied further. To determine whether the transposon had inserted clockwise or anti-clockwise of the EcoRI target of pED939 the pED939::Tn1725 plasmids were cleaved in double digestions with EcoRI and PstI; those pED939::Tn1725 plasmids which retained the 750bp EcoRI - PstI fragment of pED939 must have the transposon inserted clockwise of the EcoRI site, and therefore within the 900bp oriT fragment (see fig. 3.1). Four of the thirty-one plasmids screened had this property. From their EcoRI digests it could be ascertained that these had insertions some 400bp (pED1023), 600bp (pED1025), 750bp (pED1024) and 900bp (pED1026) from the EcoRI target of pED939 (see fig. 3A).

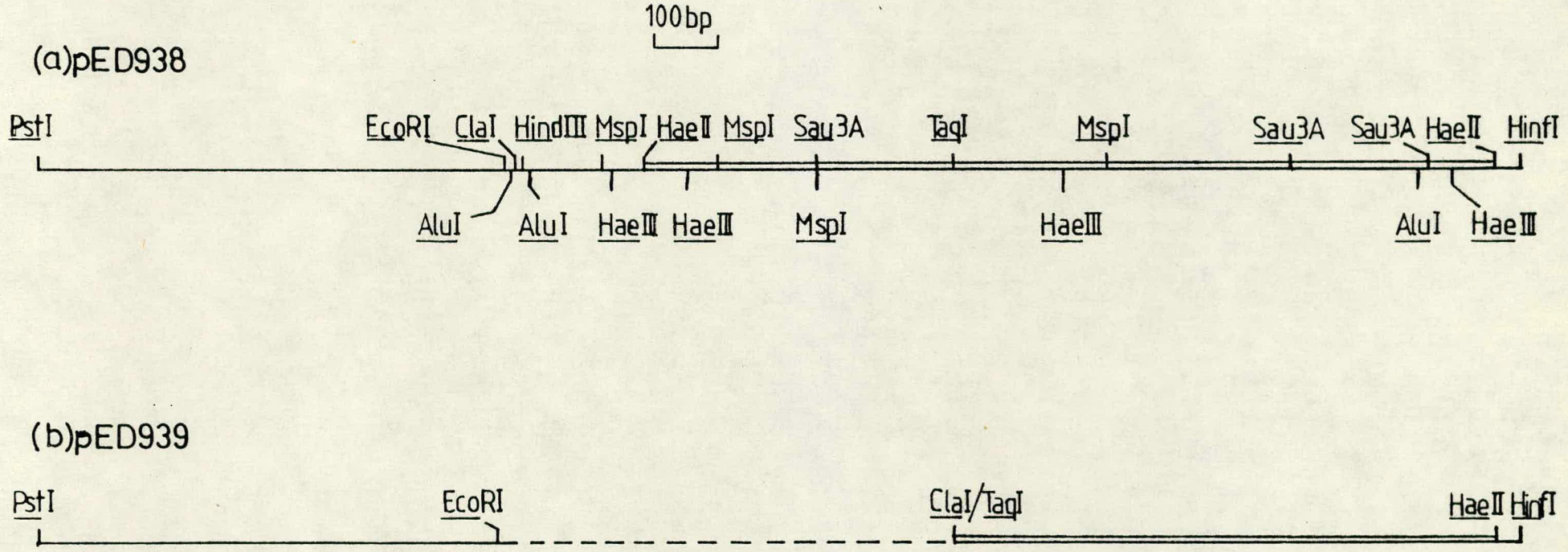
The oriT activities of pED1023, pED1024, pED1025 and pED1026 were tested. pED1029, a pSC101-based Cm^R Kan^R plasmid carrying the whole transfer region of R46 (see chapter 5) was used to supply the transfer functions. The $RecA^-$ strain JC6310 carrying pED1029 was transformed with each of the four pED939::Tn1725 plasmids

Fig. 3.1

Restriction maps of pED938 and pED939 from the unique PstI target to the HinfI target at co-ordinate 259bp on the vector plasmid pED825 (described by Everett and Willetts, 1982).

DNA derived from R46 is shown as a double line; the region deleted from pED938 to form pED939 is shown as a dotted line in fig. 3.1(b). The restriction enzyme targets present in the 750bp PstI - EcoRI fragment are not shown (see Sutcliffe, 1978; Everett and Willetts, 1982).
pED939 was derived from pED938 by deletion of a TaqI fragment (Brown, 1981).

Fig 3.1


100 bp
(a)pED938
PstI
EcoRI
ClaI
HindIII
MspI
HaeII
MspI
Sau3A
TaqI
MspI
Sau3A
Sau3A
HaeII
HinfI
AluI
AluI
HaeIII
HaeIII
MspI
HaeIII
AluI
HaeIII
(b)pED939
PstI
EcoRI
ClaI/TaqI
HaeII
HinfI

described above, or with pED939 itself as positive control. The mobilisation frequencies of these five oriT plasmids are shown in fig. 3.2. pED1024 and pED1025 gave mobilisation frequencies significantly lower than pED939; it seemed likely that in each of these plasmids (but especially pED1024) Tn1725 had inserted into DNA required for oriT activity. However, it is conceivable that the insertion of the 8.9kb transposon could prevent transfer proteins from recognising oriT even if the transposon had inserted beside, rather than within the actual oriT DNA. To minimise this uncertainty it was decided to delete most of the DNA of the transposon. The first type of deletion which was prepared removed the internal EcoRI fragment of the transposon, leaving only 30bp of the transposon (including one EcoRI target), plus the extra pED939 DNA duplicated during transposition, at the original site of insertion. A second type of deletion removed the small EcoRI target of pED939, as well as the internal fragment of Tn1725. These would effectively be derivatives of pED939 with deletions between the EcoRI targets of pED939 and Tn1725 (see fig. 3.2 and fig. 3A).

The latter deletions were made by digesting the pED939::Tn1725 plasmids to completion with EcoRI, religating at low DNA concentration, and then transforming with selection for Ap^R. Ap^R transformants were screened for Cm^R, and the plasmid DNA of those which were Ap^R Cm^S was isolated and examined on an agarose gel. Those plasmids with only one EcoRI target and one PstI target, and which when linearised co-migrated with the approximately 2kb fragment of the appropriate pED939::Tn1725 plasmid, were assumed to carry the deletions required. The deletion plasmids are shown and named in fig. 3.2.

Of these deletions, pED1039 was surprisingly difficult to isolate. The ligation conditions used should have favoured the circularisation of the EcoRI fragment containing the replication functions of pED939, with consequent deletion of both unnecessary

fragments. However, even when pED1023 had been completely cleaved with EcoRI, both small EcoRI fragments were repeatedly recovered together, such that only the fragment internal to the transposon was lost. Nineteen plasmids of this type (pED1031) were obtained before pED1039 could be isolated. A further ten plasmids containing only one EcoRI target were obtained, but these were smaller than the plasmid required, thus apparently carrying unplanned (and unexplained) extra deletions. Altogether forty-three Ap^R Cm^S recombinants were screened before pED1039 was found; far more than was necessary to find the corresponding derivatives of the other three pED939::Tn1725 plasmids. Difficulties were also experienced with pED1039 after isolation; particularly in constructing strains containing dimers of pED1039 (see section 3.h.). It is conceivable that the problems with pED1039 were related to the fact that Tn1725 had inserted, in pED1023, within a series of eleven directly repeated sequences, such that the deletion plasmid pED1039 would contain five of these repeats (see chapter 4). However, how this would affect the isolation of pED1039 is not clear.

The second class of deletion derivatives, which have lost only the internal EcoRI fragment of Tn1725 were made by partially digesting the pED939::Tn1725 plasmids with EcoRI, religating, and transforming with selection for Ap^R. A large proportion of the resulting plasmids had a size on PstI digestion very similar to that of pED939. As expected, however, these plasmids contained two EcoRI fragments, corresponding in size to the smaller fragments from the relevant pED939::Tn1725 plasmid. TaqI digestions confirmed that the two EcoRI fragments were in the same orientation as in the original pED939::Tn1725 plasmids.

This successful deletion of the 8.9kb internal fragment of Tn1725 greatly reduced the number of targets for restriction enzymes with tetranucleotide recognition sequences, so that these enzymes could be now used to map accurately the positions of the original Tn1725 insertions into pED939. Double digestions with EcoRI and

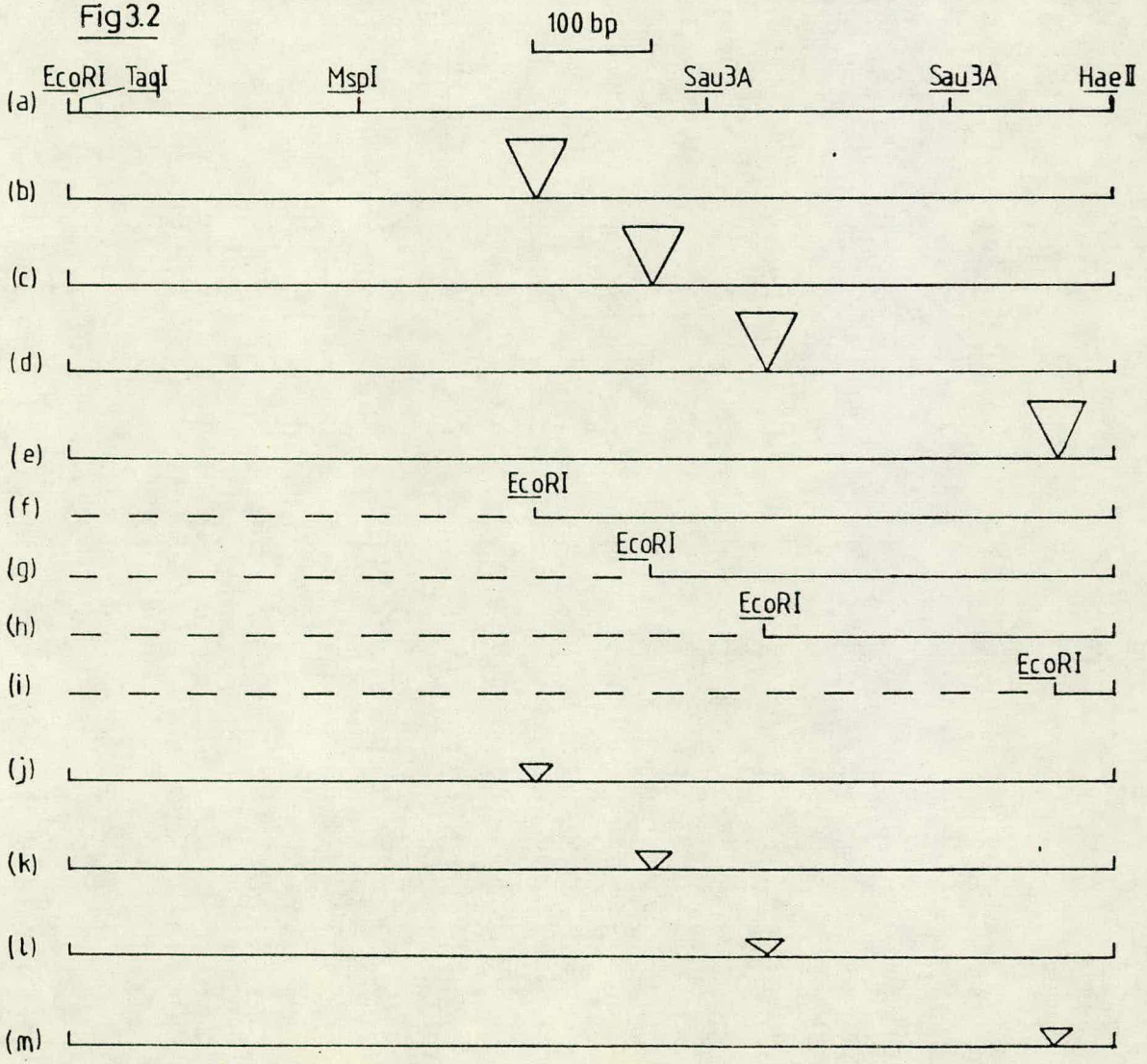
Fig. 3.2

A simplified restriction map of the 900bp TaqI - HaeII fragment of pED939 which contains oriT (a), and derivatives of pED939 which: contain Tn1725 (b-e); are deleted for segments of DNA (e-i); and have ca.35bp insertions derived from Tn1725 (j-m).

The large triangles represent Tn1725. The small triangles represent Tn1725 with the internal EcoRI fragment deleted. The dotted line represents deleted DNA.

In the conjugation experiments (see table) JC6310 containing pED1029 and the appropriate derivative was the donor strain, and ED3818 the recipient. The transfer frequency is defined as the percentage of donor cells which transferred pED1029 to the recipient. The mobilisation frequency is the number of recipients which received the oriT recombinant divided by the number which received pED1029.

Fig 3.2

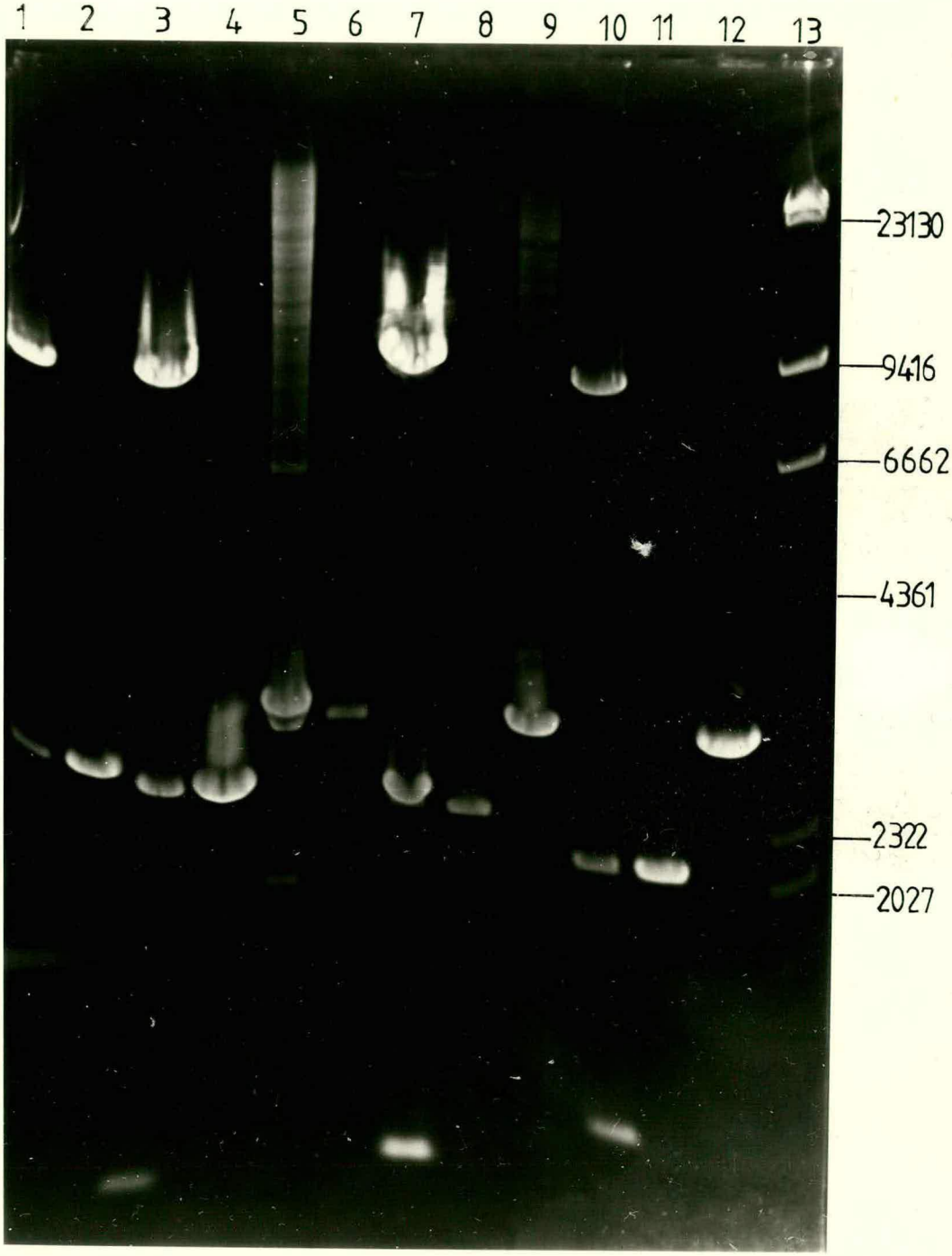


oriT plasmid	Tra,Freq. of pED1029	Mob.Freq.of oriT plasmid
pED939	22	6
pED1023	40	1.8
pED1C25	18.6	0.75
pED1024	26.8	1.5×10^{-3}
pED1026	15.8	1.9
pED1039	60	8.3×10^{-3}
pED1028	69.5	8.5×10^{-2}
pED1027	60.5	5.8×10^{-4}
pED1136	70	1.3×10^{-4}
pED1031	10.6	6
pED1033	12.1	5.1
pED1032	14.6	3.2
pED1035	6.6	5

Fig. 3.A

Restriction enzyme digestions of pED939::Tn*1725* plasmids and their derivatives.

Tracks	DNA	Enzyme
1	pED1023	EcoRI
2	pED1039	EcoRI
3	pED1025	EcoRI
4	pED1028	EcoRI
5	pED1033	PstI
6	pED939	PstI
7	pED1024	EcoRI
8	pED1027	EcoRI
9	pED1032	PstI
10	pED1026	EcoRI
11	pED1136	EcoRI
12	pED1135	PstI
13	λ7	HindIII


1
2
3
4
5
6
7
8
9
10
11
12
13
23130
9416
6662
4361
2322
2027

TaqI indicated that the distance from the TaqI target flanking the fragment of R46 DNA in pED939, to the Tn1725 insertions sites was (to the nearest ca.20bp) 400bp (pED1023), 500bp (pED1025), 600bp (pED1024) and 850bp (pED1026). The relative positions of these four Tn1725 insertions, and the structures of the deletion plasmids, have more recently been confirmed by nucleotide sequencing (see fig. 3.2, and chapter 4).

The mobilisation frequency of each of the eight deletion derivatives was measured (fig.3.2). All of the deletions which removed DNA between the transposon and the unique EcoRI target of pED939 caused a reduction in oriT activity. pED1136 showed such low mobilisation of Ap^R as to suggest that the nick site essential for transfer lies to the left of the p.o.i. - pED1026 (point of insertion of Tn1725 in pED1026; see fig. 3.2.). Admittedly mobilisation from the strain JC6310 (pED1136, pED1029), although low (1×10^{-4}), was not zero. However, examination of the plasmid DNA in four Ap^R transconjugants generated by this donor showed that pED1136 had in fact not been transferred by an oriT-dependent mechanism. The plasmid DNA was not the same size as in the donor strain; it appeared that pED1136 had recombined at low frequency with pED1029 to form a larger plasmid. There was also another plasmid present, much smaller than pED1029, but larger than pED1136. How these plasmids were formed is not understood. The donor was a recA strain, and neither parent plasmid contains transposable elements. Whatever the origin of these plasmids, their occurrence would explain the mobilisation of Ap^R of pED1136 in the absence of a nick site on pED1136.

DNA to the left of the p.o.i. - pED1023 and the p.o.i. - pED1024 is probably involved in the recognition of oriT (see figs. 3.2 and 3.7). However, this DNA is not essential, as its deletion still allowed pED1027, pED1028 and pED1039 to be mobilised with low efficiency by an oriT dependent mechanism. The essential nick site must therefore be to the right of the p.o.i. - pED1024. The frequency of mobilisation of pED1027 was low (ca. 5×10^{-4},

see figs. 3.2 and 3.7). To confirm that this was due to oriT recognition, and not to an alternative mechanism similar to that found for pED1136, the plasmid DNA from six Ap^R transconjugants was examined by electrophoresis. In five cases it was identical in size to that from the donor, as expected if mobilisation of pED1027 usually depended on recognition of its R46 oriT sequence by the homologous transfer proteins. Accordingly, if there is only one nick site in R46 it must presumably lie in the ca.250bp between the p.o.i. - pED1024 and the p.o.i. - pED1026 (see figs. 3.2 and 3.7).

All of the Tn1725 internal deletion plasmids which were effectively 35bp insertion derivatives of pED939, were mobilised at similar frequencies to pED939 (see fig. 3.2). This suggests that although 8.9kb insertions at these positions could disrupt the recognition of R46 oriT, 35bp insertions could not.

3(c) Construction of sub-clones

The Tn1725 mutagenesis, and the effects of the deletions made between the transposons and the EcoRI target of pED939, suggested that full oriT activity required DNA to the left of the p.o.i - pED1023, and between that point and the p.o.i. - pED1026. In an attempt to clone all the DNA required for oriT activity on a fragment smaller than the 900bp oriT fragment, an EcoRI - MspI fragment of pED1035 was cloned into the EcoRI and ClaI targets of pBR322. This fragment extends from the Tn1725 EcoRI target remaining in pED1035 leftward to the MspI target (see fig 3.2 and 3.3).

To construct this clone pED1135 was digested to completion with EcoRI and MspI, and pBR322 completely digested with EcoRI and ClaI. The digests were mixed and ligated overnight, then HB101 was transformed with the ligated DNA. Cloning of the EcoRI - MspI fragment required was selected by assuming that it would contain R46 oriT, and so demanding the presence of the oriT site in the recombinants. To this end the transformed HB101 cells were grown overnight in 100mls of L-broth containing ampicillin. pED1029

was then transferred by conjugation to the ampicillin resistant transformants, and the resulting HB101 (Ap^R, pED1029) transconjugants used as donors in a second filter mating with ED3818 (Rec^+ Nal^R) as recipient. After the second mating the cells were spread on plates containing ampicillin and nalidixic acid, yielding, for example, twenty colonies from 0.1ml of a 10^{-2} dilution. The plasmid DNAs from twenty-two Ap^R (Nal^R) transconjugants were isolated and sized on an agarose gel. Fourteen transconjugants contained recombinants greater in size than pBR322. The smallest was subjected to restriction endonuclease analysis. It contained an EcoRI - HindIII fragment which co-migrated on 1.2% agarose gels with the required EcoRI - MspI fragment of pED1135. The EcoRI - HindIII fragment was approximately 622bp, and as expected it was not cleaved by MspI. A map of this plasmid, pED1137, is included in fig.3.3 and its structure has been confirmed by nucleotide sequencing (see chapter 4).

JC6310 (pED1029) was transformed with pED1137 and Ap^R colonies selected. The resulting strain, JC6310 (pED1029, pED1137) was used as donor in a filter mating to ED3818. pED1137 was mobilised at a high frequency (see fig. 3.3), suggesting that all the DNA required to constitute the nick site, and for the recognition of oriT, is located within the ca.622bp EcoRI - MspI fragment (fig.3.7).

This EcoRI - MspI oriT fragment in pED1137, is inverted relative to the TaqI - HaeII oriT - fragment of pED939. Interestingly, no instability of either plasmid has been seen in the presence of pED1029, which expresses the transfer functions of R46. This is in contrast to the F oriT plasmid pED806, which in a comparable situation, is lost from 99.6% of the cells after 5 hours growth; while pED822, which contains the F oriT in the other orientation, is stable in the presence of F lac (Everett and Willetts, 1982).

The ability of pED1027 to be mobilised suggested that DNA to the right of the p.o.i. - pED1024 contains the nick site (see fig. 3.2). However, this did not rule out the possibility that there were alternative nick site(s) present in pED939 to the left

of this insertion. Deletion of these hypothetical nick sites, rather than deletion of DNA required for efficient recognition of a single nick site, could explain the low mobilisation frequency of pED1027. To test this, DNA extending from the EcoRI target of Tn1725 in pED1032 to the leftward TaqI target was cloned into pED101. pED101 is an Ap^R galK promoter-cloning vector (see section 3e). The analogous EcoRI - TaqI fragment of pED1033 was cloned into pBR322 (see figs. 3.2 and 3.3). pED1032 and pED1033 were cleaved with EcoRI and TaqI, the digests fractionated on a 5% acrylamide gel, and the required fragments extracted as described in chapter 2. The isolated fragments were mixed with pED101 or pBR322 which had been cleaved with EcoRI and ClaI, and the mixtures ligated overnight. HB101 was transformed with the ligated DNA, with selection for Ap^R. Small scale plasmid preparations were made from six Ap^R transformants in each case, and cleaved with EcoRI and HindIII. Two of the six plasmids derived from the purified pED1032 fragment were identical. They gave two EcoRI - HindIII fragments: one which co-migrated with the large EcoRI - HindIII fragment of pED101; and one which co-migrated with the EcoRI - TaqI fragment of pED1032. Similarly, six Ap^R transformants from the cloning of the EcoRI - TaqI fragment of pED1033 into pBR322 contained two EcoRI - HindIII fragments of the expected size. The structure of these clones (called pED1138 and pED1139) was confirmed by digestion with MspI, with EcoRI, and with EcoRI and TaqI; and by nucleotide sequencing in the case of pED1139 (chapter 4). Maps of these clones are shown in fig 3.3.

JC6310 (pED1029) was transformed with pED1139 and pED1138 and Ap^R was selected. The resulting strains were used as donors in matings to ED3818. Both sub-clones of the oriT region were mobilised at the same very low frequencies as pED1136 confirming the view that no nick sites are present in pED1138 and pED1139, i.e. to the left of the p.o.i. - pED1024. This region is presumably required only for efficient recognition of oriT.

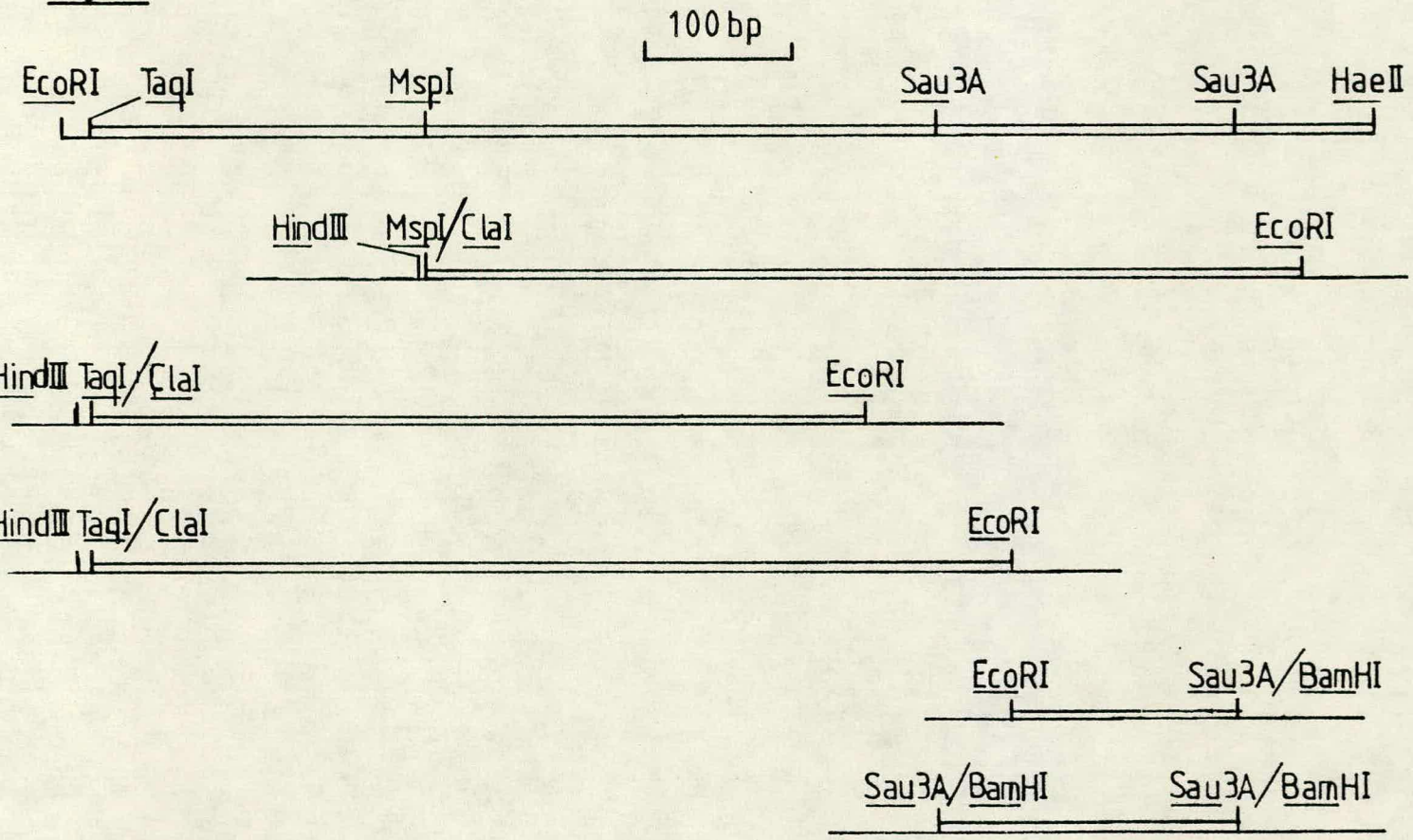
Fig. 3.3

A simple restriction endonuclease map of the TaqI - HaeII fragment of pED939 which contains oriT, and partial maps of recombinants containing fragments derived from pED939.

The maps show the DNA derived from R46 as a double line, and positioned below its original location in pED939. The neighbouring vector DNA is drawn as a single line.

In the conjugation experiments (see table) JC6310 containing pED1029 and the appropriate derivative was the donor strain, and ED3818 the recipient. The transfer frequency is defined as the percentage of donors which transfer pED1029 to the recipient, the mobilisation frequency is the number of recipients which received the oriT recombinant, divided by the number which received pED1029.

Fig3.3



oriT plasmid	Tra. freq. of pED1029	Mob. freq. of oriT plasmid
pED939	22.5	5.95
pED1137	48.5	2.85
pED1139	43	1.4×10^{-4}
pED1138	44	3.32×10^{-5}
pED1141	60.2	2.8×10^{-3}
pED967	71	1.4×10^{-3}

As explained earlier, comparison of the mobilisation frequencies of pED1136 and pED1032 suggested that the nick site was in the ca.200bp present in pED1027, but not in pED1136. The mobilisation frequency of pED967, a Sau3A clone made by Brown suggested that the essential nick site was within the 215bp Sau3A fragment (fig. 3.3). If so the site should lie within the approximately 160bp between the p.o.i. - pED1024 and the rightward Sau3A target (see fig. 3.2). To confirm this, the appropriate EcoRI - Sau3A fragment was cloned between the BamHI and EcoRI targets of pED101.

pED1027 was digested to completion with EcoRI and Sau3A and the fragments separated on an 8% acrylamide gel. The fragment required was recognised as that present in an EcoRI + Sau3A digest of pED1027, but not in that of pED939. This fragment was extracted, mixed with pED101 cleaved with EcoRI and BamHI, ligated overnight, and transformed into HB101. Four Ap^R transformants were isolated from 0.1ml of the transformation mixture. Small-scale plasmid preparations made from these colonies were digested with BamHI. Three of the four plasmids were not cleaved by BamHI. Digestion with EcoRI and PstI showed that the same three plasmids gave fragments of the expected size: one co-migrating with the larger PstI - EcoRI fragment of pED101, the other being ca.100bp smaller than the second such fragment of pED101. This was as expected if the EcoRI - BamHI fragment of pED101 had been replaced by the EcoRI - San3A fragment of pED1027. The presence of the latter fragment was confirmed by digesting one of the three recombinants with EcoRI and Sau3A, and fractionating on an 8% acrylamide gel alongside similar digests of pED101 and pED1027. The new recombinant plasmid (pED1141) clearly contained a band absent from pED101, and identical in mobility to the required pED1027 fragment.

The oriT activity of pED1141 was measured. JC6310 (pED1029) was transformed with pED1141, Ap^R cells selected, and used as donors in matings with ED3818. pED1141 was mobilised at a higher

level than pED1138, pED1139 or pED1136 (see fig. 3.3), confirming that the nick site lies in the ca.160bp region under discussion (figs. 3.2, 3.3 and 3.4).

3(d) Search for promoters near R46 oriT

The oriT of F is in an intergenic region of 292bp, with the promoters for flanking genes transcribing away from oriT (Thompson et al.,1984). The non-conjugative plasmids ColE1 and ClODF13 contain promoters which transcribe towards oriT, and it has been suggested that in these cases transcription across oriT is essential for its activity (Finnegan and Sherratt, 1982; Snijders et al., 1984). It was of interest, therefore, to know whether there are any promoters near R46 oriT which might be required for its activity, and/or for the expression of neighbouring genes. To study this, restriction fragments from the vicinity of R46 oriT were cloned upstream of a plasmid-borne galK gene which has a ribosome binding site, but no promoter (McKenney et al., 1981). The level of expression of this gene was determined for various recombinants, to obtain an indication of the position, orientation and strength of any promoters around R46 oriT.

The galK vector plasmid used was pED101 (P. Mullineaux and N. Willetts, unpublished); a restriction map is shown in fig. 3.4. There are unique restriction targets for the enzymes EcoRI, ClaI, HindIII, and BamHI upstream of the galK coding sequence. These targets can be used to clone fragments in order to assess them for promoter activity.

pED1138, already described in section 3(d) and shown in figures 3.3 and 3.4, is a pED101 derivative containing the ca.650bp of DNA to the left of the ca.160bp thought to contain the oriT nick site. The fragment is orientated in such a way that if the galK gene were expressed it would indicate the presence of a promoter transcribing towards oriT, and across the position at which Tn1725 is inserted

in pED1024. Transcription of the galK gene is measured indirectly by determining the activity of its product galactokinase, in a galK⁻ strain containing the various plasmids (see chapter 2). As shown in fig. 3.4 pED1138 did not express the galK gene.

The EcoRI - TaqI fragment from pED1028 that was cloned into pBR322 in the plasmid pED1139 was also cloned into pED101 (see figs. 3.3 and 3.4). As shown a galK⁻ strain containing this clone (pED1140) showed no more galactokinase than the plasmid-free control. These results for pED1138 and pED1139 imply that no promoter transcribes over the p.o.i. - pED1024 or the p.o.i. - pED1025, and suggest that there is no transcription towards oriT (nick site) from this (rightward) direction. However, initiation with nearby termination within the 650bp region is not excluded.

The cloning into pED101 of the 160bp EcoRI-Sau3A fragment, shown in section 4(d) to contain the nick site, was described earlier. The galactokinase levels produced by this plasmid, pED1141 (fig 3.4), suggest that no promoter transcribes leftward across the position at which Tn1725 is inserted in pED1024 (see fig. 3.2).

There is, therefore, no evidence from pED1138, pED1139 and pED1141 for a promoter transcribing rightwards towards, or leftwards from within the 160bp region known to contain the nick site of R46. The 160bp thought to contain the nick site is only ca.100bp from the HaeII target at the junction between the R46 sequences and those of the vector. It is therefore possible that a vector promoter transcribing leftward across the HaeII target could compensate for a promoter normally active in R46. This hypothetical R46 promoter would be outside the 900bp oriT fragment, and so not cloned in any of the pED101 derivatives. However, it is unlikely that such a vector promoter could transcribe across the nick site in pED1026, in which Tn1725 has inserted between the HaeII target and the nick site. It is also unlikely that a promoter beyond the HaeII target could transcribe across the nick site in R46, as transcription from it would converge with transcription from the R46 promoter shown below.

Fig. 3.4

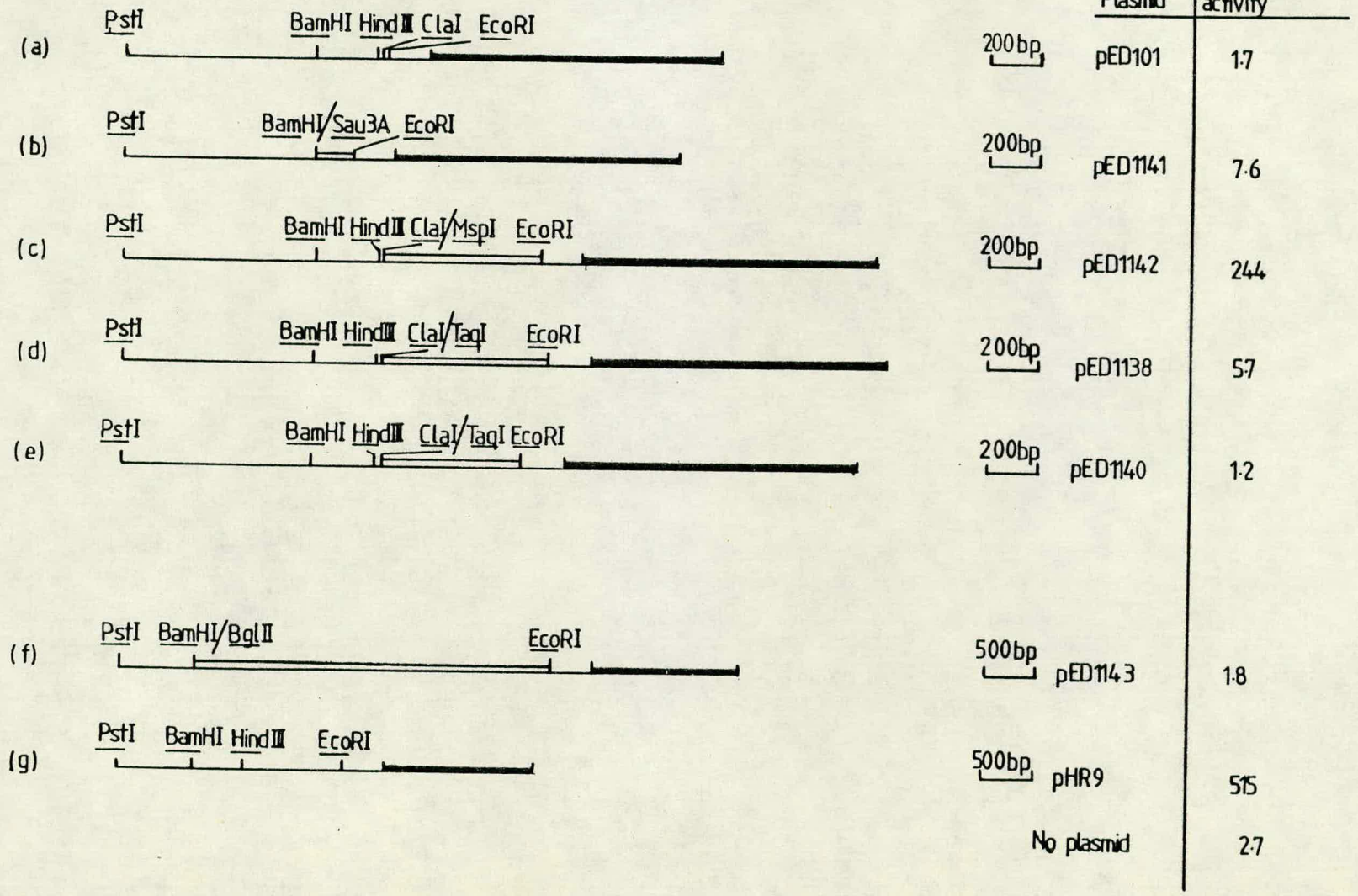
Restriction maps of the recombinants used to detect promoter activity in the vicinity of R46 oriT. Only the region from the unique PstI target in the Ap^R gene, to the end of the galK coding sequence is shown for each plasmid.

The sources of the fragments which are shown here cloned into pED101 are illustrated in fig. 3.3 (for pED1141, pED1142, pED1138 and pED1140) and in fig. 3.5 (for PED1145). The DNA derived from R46 is shown as a double line, the DNA which contains the galK coding sequence is a solid line, and the neighbouring vector DNA as a single line.

pHR9 contains the promoter of the gal operon linked to the galactokinase gene (Newman et al., 1982).

Galactokinase activity is measured as nanomoles galactose phosphorylated per minute per O.D.650 (see chapter 2).

Fig3.4


(a)
PstI
BamHI HindIII ClaI EcoRI
200bp
pED101
1·7
(b)
PstI
BamHI/Sau3A EcoRI
200bp
pED1141
7·6
(c)
PstI
BamHI HindIII ClaI/MspI EcoRI
200bp
pED1142
24·4
(d)
PstI
BamHI HindIII ClaI/TaqI EcoRI
200bp
pED1138
5·7
(e)
PstI
BamHI HindIII ClaI/TaqI EcoRI
200bp
pED1140
1·2
(f)
PstI BamHI/BglII EcoRI
500bp
pED1143
1·8
(g)
PstI BamHI HindIII EcoRI
500bp
pHR9
515
No plasmid
2·7
Plasmid
Galactokinase activity

The smaller EcoRI - HindIII fragment of pED1137, which contains the ca.622bp EcoRI - MspI fragment known to include all DNA necessary for oriT activity, was also cloned into pED101 (see figs. 3.3 and 3.4) by digesting pED1137 with EcoRI, HindIII and MspI, mixing with pED101 cleaved with EcoRI and HindIII, ligating overnight, and transforming into HB101. The transformed cells were then grown in 100mls of L-broth containing ampicillin, and Ap^R $oriT^+$ plasmids were selected in the way described for the construction of pED1137 (see section 3(d)). Restriction analyses confirmed that these Ap^R $oriT^+$ plasmids contained the EcoRI - HindIII fragment of pED1137 cloned into pED101.

One such plasmid, pED1142, was introduced into the $galK^-$ strain JC3272. Single colonies of JC3272 (pED1142) appeared white on galactose tetrazolium plates, which suggested they were expressing galK. This was confirmed by galactokinase assays (fig. 3.4). There is a relatively strong promoter transcribing rightwards across the p.o.i. - pED1026, and away from oriT, (fig. 3.7). As there was no evidence for a promoter in the EcoRI - TaqI fragment cloned in pED1138, this rightward promoter must be positioned in the ca.200bp between the p.o.i. - pED1024 and the p.o.i. - pED1026. It could therefore either transcribe across the nick site from a position near Tn1725 in pED1024, or away from the nick site from a point to its right. These alternatives could be distinguished by fusing to galK smaller restriction endonuclease fragments, suitably chosen and orientated, but this was not attempted. However, the sequence within this region most closely related to the promoter consensus (Hawley and McClure, 1983) is positioned between the Sau3A target and Tn1725 in pED1026 (see chapter 4). This would not cause transcription across the nick site, but rightwards away from it, (fig. 3.7). The results presented in section 3(f) orientate the cloned TaqI - HaeII fragment containing R46 oriT in R46, and suggest that the promoter described above transcribes towards the transfer genes, and might therefore be required for expression of one or more of these.

To determine whether transcription in this direction continued for at least some 700bp into the transfer region, a 3.7kb EcoRI - BglII fragment was cloned between the EcoRI and BamHI targets of pED101. This insert contains most of the 4.6kb BglII fragment carrying oriT, and includes approximately 600bp of the region thought to encode the transfer genes. This EcoRI - BglII fragment was cloned into pED101 in the same as was used (section 3(f)) to clone it into pBR322 (figs. 3.4 and 3.5). Galactokinase assays of a galK$^-$ strain containing this recombinant (pED1143) show that it does not express galK. This implies that the transcription seen to emerge from the oriT DNA inserted in pED1142 must be terminated within 700bp beyond the point corresponding to the EcoRI site in pED1142.

3(e) The position and orientation in R46 of the TaqI - HaeII fragment containing oriT

The exact position of the TaqI - HaeII fragment containing oriT within the transfer region of R46 would allow the oriT$^+$ clones to be related to R46 itself. Further, to correlate the orientation of transfer of the oriT clone pED1137 (section 3(g)) to R46 it was essential to determine the orientation in R46 of the cloned sequences.

Brown showed that the HaeII fragment cloned in pED938, which contains oriT, originated from the BglII - F fragment of R46 (Brown, 1981). Langer and Walker (1981) showed that this BglII fragment contained a 2.7kb HpaI fragment. According to the data of Langer and Walker the HpaI fragment was placed symmetrically within the 4.6kb BglII fragment such that each HpaI fragment was 0.9kb from a BglII site (see fig. 3.5(a)). As the 1.4kb HaeII fragment cloned in pED938 was derived from the BglII - F fragment, but contains no BglII or HpaI targets, it can be deduced that it originated within the 2.7kb HpaI fragment. This was confirmed by using ^{32}P-labelled pED938 as a probe in a Southern blot hybridisation against R46 digested with HpaI. As expected, pED938 hybridised only to the 2.7kb HpaI fragment.

It would have been possible to clone the BglII - F fragment, or the 2.7kb HpaI fragment of R46, and to construct a restriction map of the recombinants. However, the restriction map of such a recombinant could not have been used to locate and orientate oriT on R46, as it would have been impossible to deduce the orientation of the cloned BglII or HpaI fragment in R46. To overcome this problem an R46::Tn1725 plasmid was used. Thirty transfer-deficient Tn1725 insertions into R46 were made (see chapter 5). In three of these, insertion was within the BglII - F fragment. The position of one such insertion is shown in fig. 3.5(a). All three plasmids (pED1036, pED1038 and pED1146) contained the 2.7kb HpaI oriT^{+} fragment within an EcoRI - BglII fragment. Cloning and restriction mapping of this EcoRI - BglII fragment would allow the information gathered from pED938 and its derivatives to be related to R46.

Firstly, to reduce the size of the R46::Tn1725 plasmid pED1036, in which Tn1725 had inserted nearest to the HpaI fragment, it was digested to completion with HindIII, religated in a dilute solution, transformed into HB101 and the transformed cells spread on an ampicillin plate. Those Ap^{R} transformants which were Ap^{R} Tc^{S} Cm^{S} were studied further. EcoRI + BglII digestion of small scale DNA preparations confirmed that the plasmids in these transformants consisted of DNA extending from the HindIII target within Tn1725 of pED1036 to that at co-ordinate 49kb on the R46 map (see figs. 3.5 and 1.1). One of these plasmids was called pED1144 (see fig. 3.5). Sub-cloning of the oriT - containing EcoRI - BglII fragment of pED1144 was now relatively easy, because pED1144 contains only 19kb compared to the 60.6kb of pED1036, and includes only three EcoRI - BglII fragments.

pED1144 was cleaved to completion with EcoRI + BglII and pBR322 digested to completion with BamHI and EcoRI. 3ug of the former and 1ug of the latter digest were ligated overnight. To enrich for the desired recombinant the DNA was ethanol precipitated after ligation, and cleaved with HindIII + BamHI + BglII. These three enzymes would not cleave the desired recombinant, but they

would cleave pBR322 and a number of other possible recombinants. The DNA was then transformed into HB101, and the products grown in 100mls of L-broth containing ampicillin. oriT - containing recombinants were selected as described for pED1137 (section 3(d)). Plasmid DNA was isolated from twelve Ap^R (Nal^R) transconjugants and cleaved with EcoRI + SalI. One of the plasmids, pED1145, had the restriction pattern expected if the EcoRI - BglII fragment of pED1144 had been cloned between the EcoRI and BamHI targets of pBR322. Its structure was confirmed by HpaI and EcoRI digestions.

As explained earlier the information gathered from this recombinant can be related to R46, as the orientation in R46 of the cloned EcoRI - BglII fragment is known. Therefore the position and orientation in R46 of the TaqI - HaeII fragment which contains oriT could be determined from a restriction map of pED1145.

To construct such a map, pED1145 was cleaved with a number of enzymes. Cleavage with HinfI showed that oriT must be within the largest fragment (2100bp) because there are no HinfI targets within the 1400bp HaeII fragment containing oriT (see fig. 3.1). The oriT - containing HinfI fragment must therefore be larger than 1400bp, and the only such fragment was 2100bp (fig. 3.5(e)). Similarly R46 oriT was shown to be within a 1275bp TaqI fragment, as this is the only R46 TaqI fragment in pED1145 larger than 900bp (fig. 3.5(e)). When pED1145 was cleaved with HinfI plus TaqI the largest fragment was 1050bp. This must be derived from the 1275bp TaqI fragment and the 2100bp HinfI fragment, as these are the only fragments large enough to produce it. Furthermore, it is the only TaqI - HinfI fragment larger than 900bp, and therefore, from the restriction map of pED938 (fig. 3.1), must contain oriT. These data plus the restriction map of pED938 (fig. 3.1) allowed a map of a ca.2250bp region of pED1145, which contains oriT, to be constructed (fig. 3.5(e)). The whole 2250bp region shown in fig. 3.5(e) must lie within the 2.7kb HpaI R46 fragment, as the 2100bp HinfI and 1275bp TaqI fragments are not cleaved by HpaI.

Fig. 3.B

Restriction analysis of pED1145.

Track	DNA	Enzyme
1	pED1145	TaqI
2	pBR322	TaqI
3	pED1145	HinfI
4	pBR322	TaqI
5	pBR322	TaqI
6	pED1032	EcoRI
7	pED1145	HincII
8	pED1145	HpaI
9	pED1145	HincII (partial digestion)
10	pBR322	TaqI
11	λ7	HindIII

Tracks 1-4 were run on a 2% agarose gel; tracks 5-7 on a 1.7% agarose gel and tracks 8-11 on a 1% agarose gel.

TaqI digested pBR322 produced size standards including a fragment of 616bp. There is not a 616bp TaqI fragment in pBR322, but it appears due to methylation of the TaqI target at co-ordinate 1126bp, with consequent fusing of the pBR322 fragments of 475bp and 141bp.

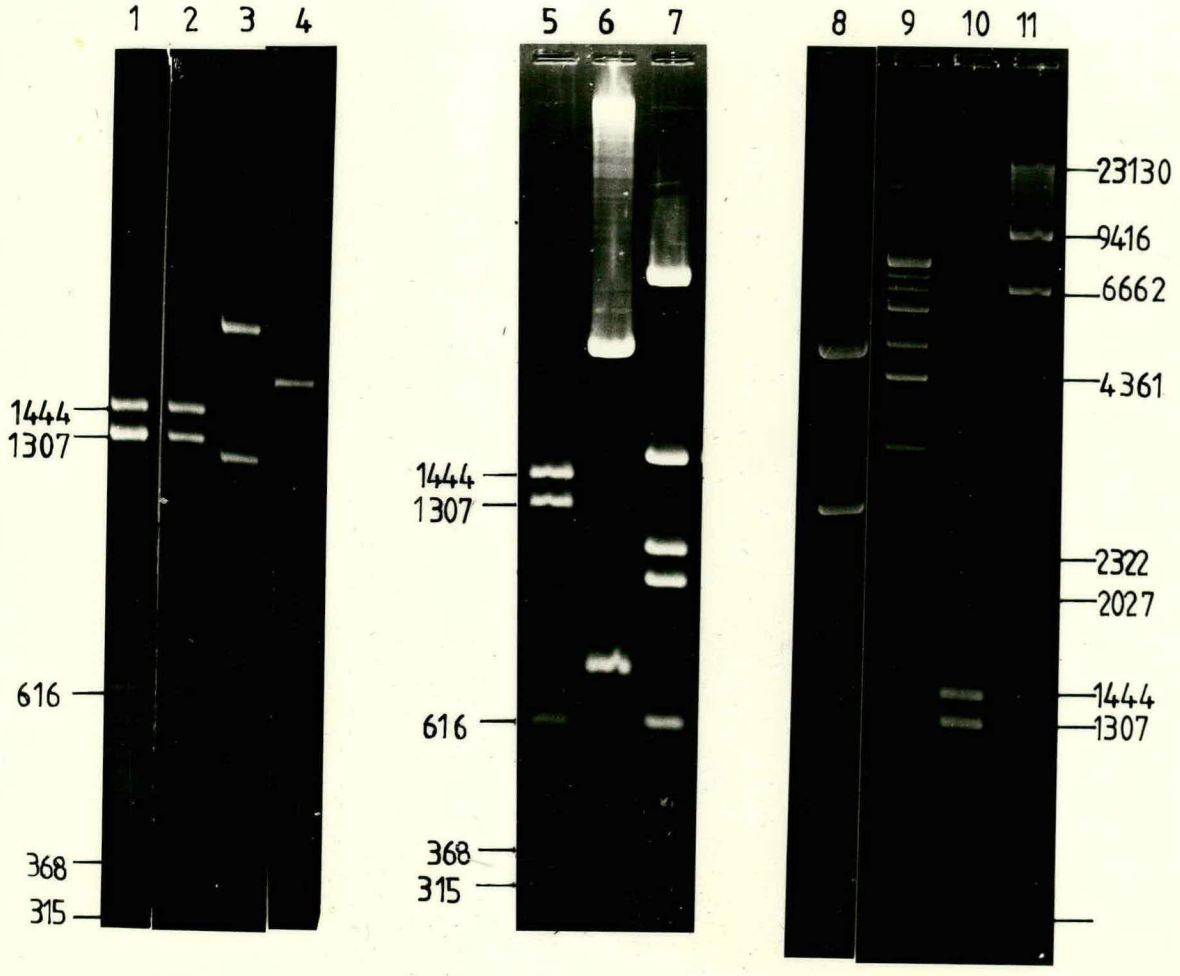

1
2
3
4
5
6
7
8
9
10
11
1444
1307
616
368
315
1444
1307
616
368
315
23130
9416
6662
4361
2322
2027
1444
1307

Fig. 3.5

Restriction maps of the plasmids used to determine the orientation in R46 of the TaqI-HaeII fragment of pED939 which contains oriT.

DNA derived from R46 is drawn as a double line; Tn1725 is represented as a line with a circle on top (3.5(a)), and DNA derived from it as a solid box (3.5(b)); vector DNA is drawn as a single line. The approximate location of the nick site is shown as an arrow (3.5(e)).

(a) illustrates pED1036 linearised at the HindIII target at co-ordinate 48.95kb on the R46 map (fig. 1.1). This map only shows the relevant BglII, HindIII, EcoRI and HpaI targets.

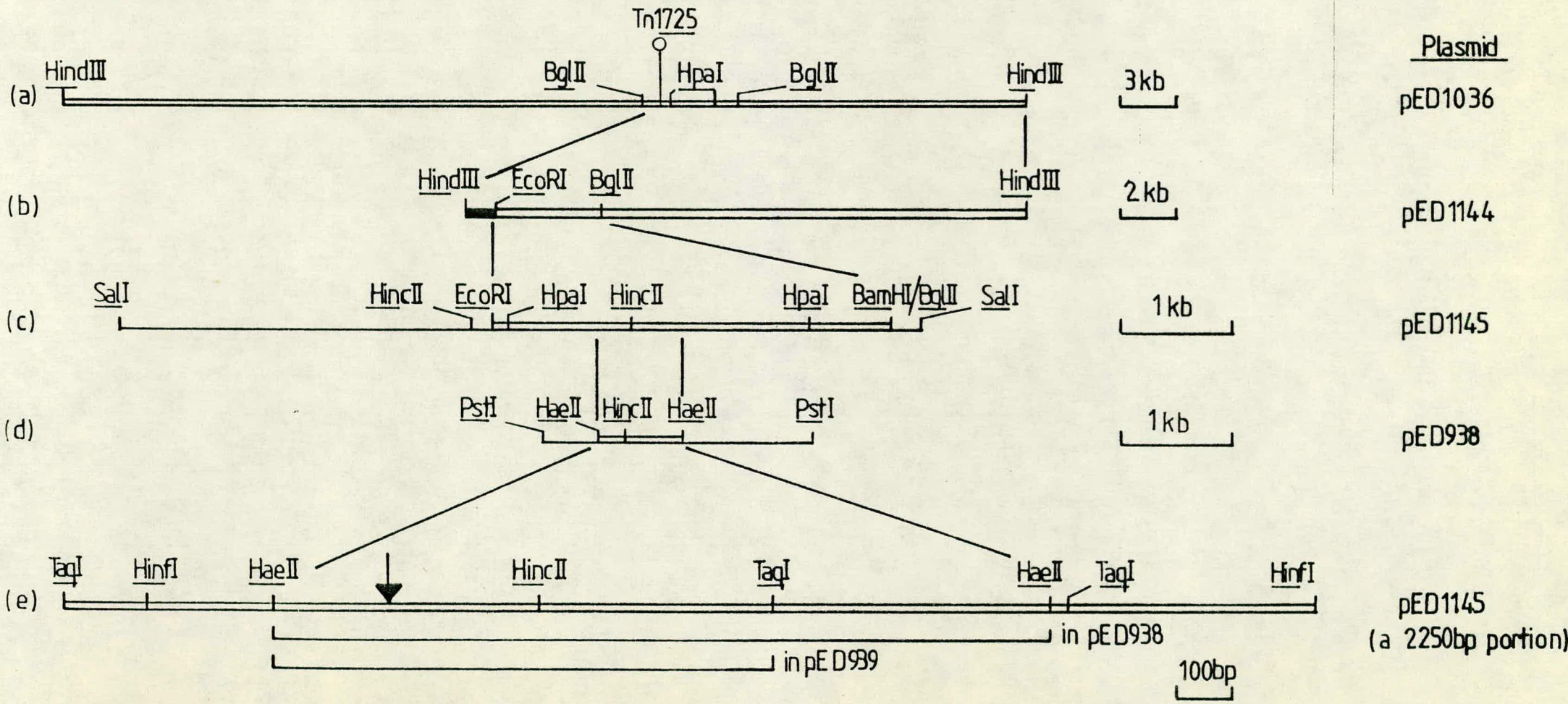
(b) illustrates pED1144 linearised at its unique HindIII target, showing only the relevant EcoRI and BglII targets. The 3.7kb EcoRI - BglII fragment contains oriT.

(c) illustrates pED1145 linearised at its unique SalI target. All the EcoRI and HpaI targets are shown, and all the HincII targets additional to the SalI and HpaI targets (which are also HincII) are shown.

(d) The HaeII fragment of pED938 which contains oriT is positioned directly below its location in pED1145.

(e) a restriction map of a 2250bp region containing oriT. This was constructed as described in the text. There may be other HaeII targets outwith the 1400bp HaeII fragment, and other TaqI targets within the 450bp TaqI - HinfI fragment on the right hand side of the map.

Fig 3.5


Tn1725
Plasmid
(a)
HindIII
BglII
HpaI
BglII
HindIII
3kb
pED1036
(b)
HindIII
EcoRI
BglII
HindIII
2kb
pED1144
(c)
SalI
HincII
EcoRI
HpaI
HincII
HpaI
BamHI/BglII
SalI
1kb
pED1145
(d)
PstI
HaeII
HincII
HaeII
PstI
1kb
pED938
(e)
TaqI
HinfI
HaeII
HincII
TaqI
HaeII
TaqI
HinfI
pED1145
(a 2250bp portion)
in pED938
in pED939
100bp

The 2.7kb HpaI fragment contains a single target for HincII which also lies within the TaqI - HaeII (oriT) sub-fragment cloned in pED939 (see sequence in chapter 4; and fig. 3.5). The HincII target is 480bp from the HaeII end, and 420bp from the TaqI end of the latter fragment. HincII is an enzyme with a partially degenerate recognition sequence (GTPyPuAC) and will cleave at HpaI sites (GTTAAC). HincII digestion of the 2.7kb HpaI fragment produced two fragments of about 1600bp and 1130bp. The HincII target also lies in the 2100bp HinfI fragment, containing oriT; 1400bp from one HinfI terminus: for the HinfI fragment yields 1400bp and 700bp sub-fragments when cut with HincII (and it is not cut by HpaI). Only the 1600bp HincII segment of the 2.7kb HpaI fragment is, then, large enough to accommodate the 1400bp HincII - HinfI fragment.

As shown in fig. 3.5(e) the 1400bp HincII - HinfI fragment contains the 420bp HincII - TaqI fragment of pED939 which must therefore map within the 1600bp HincII fragment. Consequently, the 480bp HinII - HaeII fragment of pED939, which has been shown to contain the nick site of R46 (section 3(d)), must be located within the 1130bp HincII fragment.

To position and orientate in R46 the TaqI - HaeII fragment containing oriT, all that remains to be determined is the order of the 1130bp and 1600bp HincII fragments within the approximately 2.7kb HpaI fragment. This was done by partially cleaving pED1145 with HincII. pED1145 contains five HincII fragments: 3256, 1600, 1130, 1000 and 630bp. The 3256bp fragment is from pBR322, the 630 and 1000bp fragments flank the 2.7kb HpaI fragment (as shown in fig. 3.5(c)), and the 1600 and the 1130bp fragments are from within the HpaI fragment.

The order of the two HincII fragments within the HpaI fragment can be determined by measuring the size of the fragments in a partial HincII digest. The only fragments of less than 2.7kb which were present when pED1141 was cleaved partially with HincII, but absent from the complete digest, were approximately 1800bp and

2600bp in length. There were clearly no fragments of 2000 and 2150bp (fig. 3.B.). This result unambiguously positioned the 1600bp HincII fragment, from within the 2.7kb HpaI fragment, beside the 1000bp flanking fragment and not beside the alternative 650bp flanking fragment: moreover 650bp + 1130bp explains the 1800bp partial digestion product.

From these data it was concluded that the TaqI and HaeII targets used to clone R46 oriT in pED939 map at R46 co-ordinates 14.8kb and 15.7kb respectively (see fig. 3.5(a) (b) (c) (d)).

3(f) The direction of transfer of R46

The direction of transfer of R46 was determined by utilising regions of homology between plasmids containing R46 oriT, and the chromosome of the host strain. This homology allowed the plasmid to integrate reversibly into the E.coli chromosome, and therefore to promote transfer of chromosomal markers. Studies of the direction of transfer of chromosomal markers flanking the area of homology accordingly allowed the direction of transfer of the plasmid to be deduced.

Two E.coli strains, CE60-103 and CE60-124, with a copy of Tn5 inserted in the lactose operon, were used as donors in these matings. To create homology between Tn5 and the R46 oriT plasmid pED1137 a 3.4kb HindIII fragment internal to Tn5, and which contains the Kan^R marker of Tn5, was cloned into the unique HindIII target of pED1137. An R46::Tn5 plasmid, pED957, was used as a source of this Kan^R fragment. The resulting recombinant was called pED1112.

The absolute orientations of the Tn5::lac insertions in CE60-103 and CE60-124 was not known. It would, therefore, not have been possible to interpret the results obtained for transfer driven by integrated pED1112, without a suitable control. To provide such a control a plasmid was constructed which contained homology with Tn5, and for which the direction of transfer was known, by cloning the HindIII fragment of Tn5 into the HindIII target of the F oriT clone pED822. The resulting plasmid was called pED1113

(see fig. 3.6). The direction with which pED1113 promoted transfer of the chromosomal markers flanking the *lac* genes of CE60-103 and CE60-124 could be related to the known direction of transfer of pED1113 itself. Therefore the direction of transfer of pED1112 could be deduced from a comparison of pED1112 - and pED1113 - promoted transfer of the chromosomal markers. However, it was first necessary to determine the orientations of the *HindIII* fragment of *Tn5* in pED1113 and pED1112. This was achieved by cleaving both plasmids with *PstI*; there is an asymmetrically located *PstI* target within the 3.4kb *HindIII* fragment, and one within the Ap^R gene of both plasmids (see fig. 3.6). The restriction data showed that the fragment in pED1112 is in the opposite orientation to that in pED1113.

pED1112 and pED1113 were transformed into CE60-103 and CE60-124, and Ap^R colonies selected. These *oriT* clones require the transfer genes of R46 or F, respectively, in order to transfer. A derivative of the F plasmid, pED100 (Willetts and Johnson, 1981), was used to supply the transfer functions of F. This plasmid contains no insertion sequences, and is not itself capable of transferring the bacterial chromosome. Similarly, pED912 (Brown and Willetts, 1981) was used to supply the transfer functions of R46. pED100 and pED912 were transferred into the strains containing pED1113 and pED1112, respectively. The four resulting strains were used as donors in matings and the frequencies of transfer of chromosomal markers measured.

As shown in fig. 3.6 the *lac* operon of *E.coli* is flanked by the genes *proA* and *purE*. The only chromosomal mutation present in CE60-103 and CE60-124, apart from the *Tn5* insertion, is a *trpE* deletion; they are accordingly potential $proA^+$ or $purE^+$ donors. A *proA purE* double mutant was not available, so two separate recipients were used. These were X478 (*purE* Sm^R) and AB1157 (*proA* Sm^R). The genotypes of these strains are presented in table 2.1.

The four strains containing the plasmids were used as donors to X478 and AB1157. The transfer frequency of the Ap^R *oriT* containing clone, and of the chromosomal marker appropriate to the recipient used, was determined in each case. The results of these filter matings are shown in fig. 3.6.

The direction in which pED1112 and pED1113 promoted chromosomal transfer from CE60-103 was opposite to that applying in CE60-124. Evidently Tn*5* is inserted in opposite orientations in CE60-103 and CE60-124. Further pED1113 promoted a higher level of transfer of the *purE* marker of CE60-103, while pED1112 promoted transfer of its *proA* marker. Similarly pED1113 transferred the *proA* marker of CE60-124 at a high level while pED1112 promoted *purE* transfer. Since the *Hind*III fragment of Tn*5* is oriented in opposite directions in pED1112 and pED1113, this implies that the transfer directions of pED1112 and pED1113 are the same. Accordingly the R46 *oriT* cloned in pED1136 transfers the plasmid in a clockwise orientation similar to that of F *oriT* in pED1113 (see fig. 3.6).

The data described in section 3(f) orientate the $oriT^+$ *Eco*RI-*Msp*I fragment cloned in pED1136, with respect to R46. Since pED1136 transfers as described above, then R46 must transfer anti-clockwise as drawn (figs.1.1, 3.6).

The R46::Tn*5* plasmid pED957 was also used to promote transfer of the chromosome of CE60-103 and CE60-124. The Tn*5* insertion in this plasmid had been located at co-ordinate 37.15kb on the R46 map (see fig. 1.1) by A. Brown (1981). The orientation of the Tn*5* insertion in pED957 was determined by *Bam*HI and *Bam*HI plus *Sma*I digestion. There are single *Sma*I and *Bam*HI targets within Tn*5*, and a *Bam*HI target at co-ordinate 44kb in R46. The *Bam*HI fragment extending from the target within Tn*5* to co-ordinate 44kb in R46 was cleaved with *Sma*I. Thus Tn*5* is orientated in pED957 as shown in fig. 3.6.

CE60-103 and CE60-124 were transformed with pED957, and Ap^R transformants selected. The resulting CE60-103 (pED957) and CE60-124 (pED957) strains were used as donors. X478 could not be used as recipient in these matings as the low level Sm^R gene of pED957 prevented adequate contraselection. For this reason JE2571, which is a galK$^-$ strain, was used as a recipient instead of X478 (see fig. 3.6). pED957 was shown to transfer proA more effectively than galK from CE60-103, and to transfer galK, but not proA from CE60-124. This is as expected if R46 transfers its transfer genes last.

3(g) Recombination of pED1039 during mobilisation

In Rec^+ cells two or more copies of a given cloning vector will commonly recombine to form dimers or multimers (see Summers and Sherratt, 1984). It has been show that dimers of recombinants containing the oriT of ColE1, F or RSF1010 are resolved to monomers during mobilisation (Warren and Clark, 1980; Everett and Willetts, 1982; K. Derbyshire, 1983). This process can be explained in two ways: by early nicking at both oriT sites of the dimeric molecule in the donor, with consequent mobilisation of a monomer; or by nicking at a single oriT site, initiating transfer of a dimer which later forms a monomer because the leading end of the transferred oriT can recognise the incoming uncleaved oriT site, and recombine with it to yield a monomeric circle. The above findings have been cited as evidence for strand breakage and re-union at oriT during transfer. The R46 oriT clone pED1039, was studied to determine if it behaves in a similar way to the oriT clones of ColE1, F and RSF1010.

It was first necessary to ensure that pED1039 formed dimers. Undigested pED1039 DNA, which had been isolated from the Rec^+ strain ED8654 was run on an agarose gel. There appeared to be two forms of the plasmid, whose mobilities relative to pBR322 suggested that they represented monomeric and dimeric circles.

Fig. 3.6

Restriction maps of the plasmids used to determine the direction of transfer of R46, and a schematic diagram indicating the relative positions of the chromosomal markers mentioned in section 3.f.

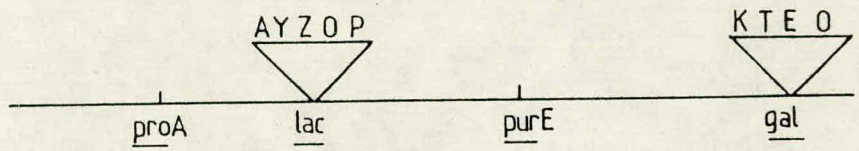
(a) The relative positions of the proA, lac, purE and gal markers on the E.coli chromosome.

(b) Restriction maps of the three plasmids carrying Tn5 (pED957) or a fragment of Tn5 (pED1112 and pED1113), are shown. The orientation of Tn5 is demonstrated by the position of the PstI target within the HindIII fragment. The known direction of transfer of pED1113 and the direction of transfer of pED1112 and pED957 (from this experiment) are shown by the arrows on the restriction maps.

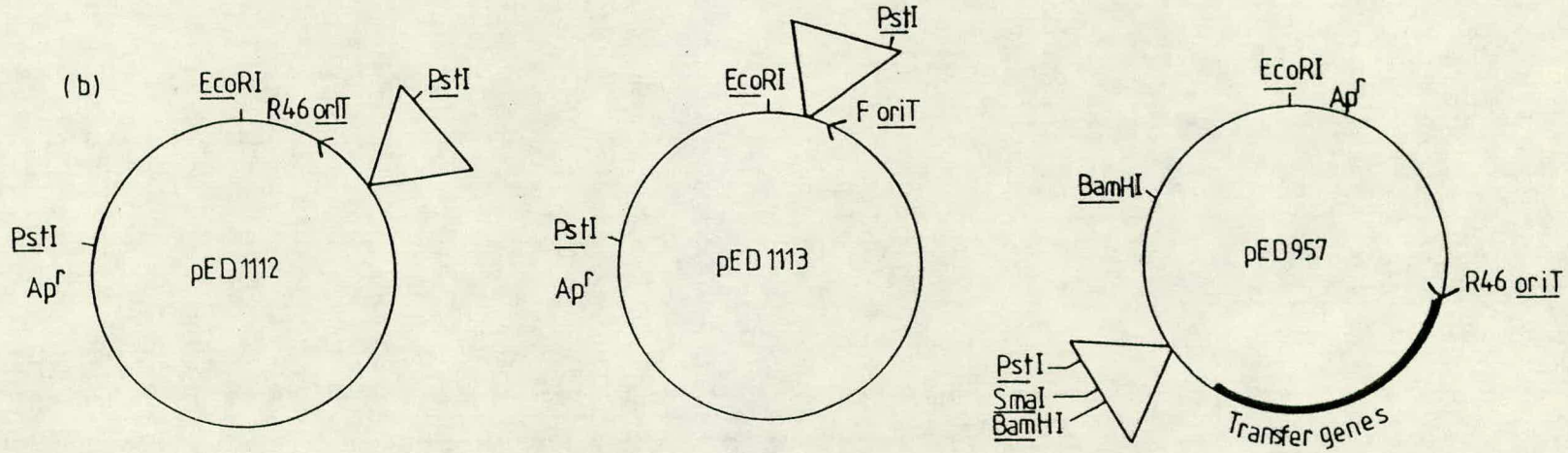
(c) The figures represent the numbers of recipients/ml of resuspended mated cells (see method for filter matings, chapter 2) which have inherited either the chromosomal marker or the plasmid (Ap^R). The small number of revertants found in each case have been subtracted. pED100 or pED912 alone in the donors did not promote transfer of chromosomal markers from either strain.

Fig3.6

(a)



(b)



(c)

Donor \ Recipient	AB1157		478	
	Ap^R Transf.	proA Transf.	Ap^R Transf.	purE Transf
CE60-103 (pED100,pED1113)	2×10^7	2.5×10^1	1.35×10^7	9.25×10^2
CE60-124 (pED100,pED1113)	3.1×10^7	5.6×10^2	1.5×10^7	0
CE60-103 (pED912,pED1112)	3.1×10^7	4.4×10^2	5.6×10^7	0
CE60-124 (pED912,pED1112)	2.5×10^7	0	1.2×10^8	1.05×10^3

Donor \ Recipient	AB1157		JE2571	
	Ap^R Transf.	proA Transf.	Ap^R Transf	galK Transf.
CE60 - 103 (pED957)	2.7×10^8	5.6×10^2	2.5×10^8	10^2
CE60 - 124 (pED957)	3.6×10^8	0	2.7×10^8	1.6×10^3

JC6310 (recA$^-$), containing pED1029 to provide transfer functions, was transformed with pED1039, and Ap^R transformants selected. The plasmid DNAs were isolated from twenty-three transformants derived from two independent transformations, and run on an agarose gel. The pED1039 DNA used for the transformation acted as a control. None of the transformants contained DNA co-migrating with the covalently closed circular (CCC) monomer or dimer pED1039 DNA used for the transformation. One transformant appeared to contain a deletion derivative of pED1039, as the plasmid DNA migrated ahead of CCC monomeric pED1039 DNA. The twenty-two other transformants contained plasmids which appeared to be identical, and migrated slower than monomeric CCC pED1039, but ahead of CCC dimeric pED1039. When these plasmids were cleaved with EcoRI they gave a single band of 3.6Kb, which is 1.2Kb larger than linear pED1039. This plasmid could be mobilised by pED1029. This behaviour of pED1039 has not been explained. Note that problems had also been experienced in the original isolation of pED1039 (see section 3(d)).

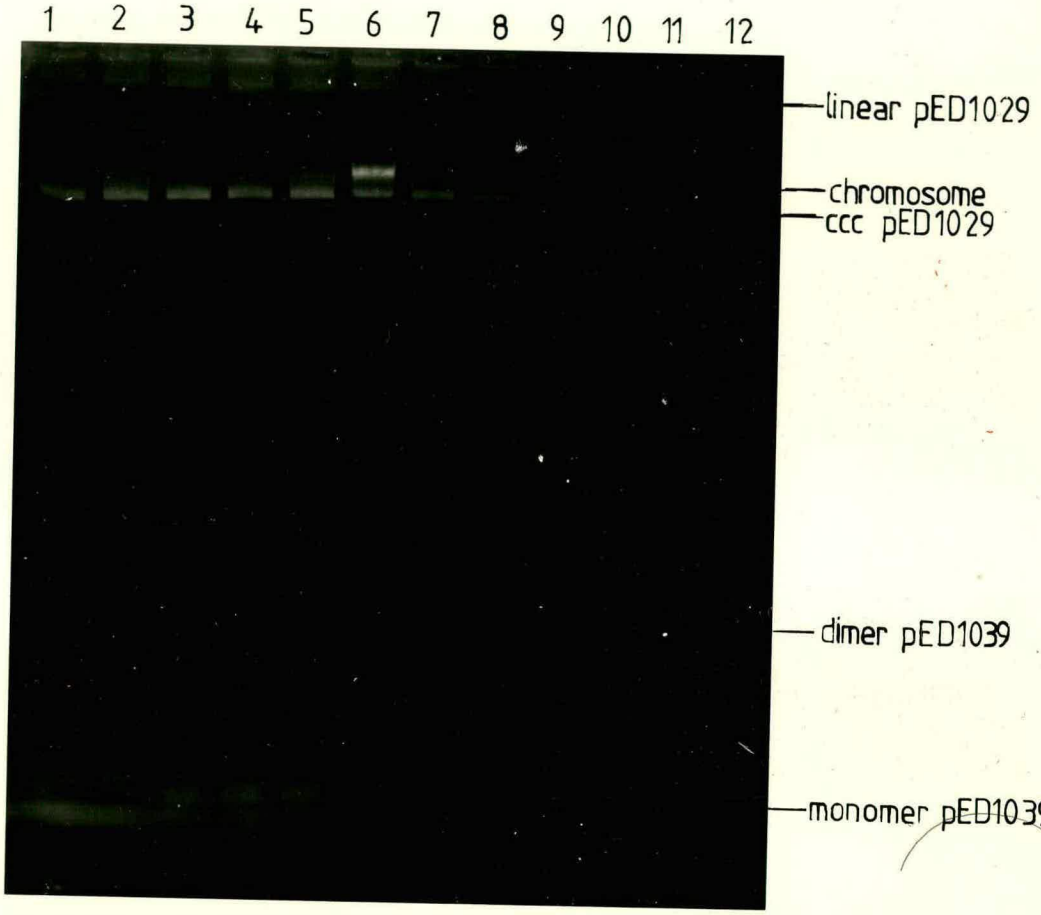
In an attempt to avoid these problems JC6310 without the resident pED1029 was transformed with pED1039, and Ap^R selected. Plasmid was isolated from eight of the resulting Ap^R transformants, and run on an agarose gel, with the pED1039 DNA used for transformation as control. When the plasmid DNA from the transformants was cleaved with EcoRI it appeared to be the same size as linear pED1039 DNA. The undigested plasmid DNA from seven transformants appeared to be monomeric, while the eighth was dimeric. The JC6310 (pED1039 (dimer)) strain was then used as a recipient for pED1029 and Kan^r transconjugants selected. The DNA was isolated from the resulting JC6310 (pED1039(dimer)) transconjugants and run on an agarose gel. This confirmed that the pED1039 DNA was as in the original JC6310 (pED1039(dimer)) strain. JC630 (pED1039 (dimer), pED1029) was then used as donor in matings with the recA strain ED3886. ED3886 is Spc^R, so Ap^R (Spc^R) transconjugants were selected. Plasmid DNAs isolated from seventy-three Ap^R (Spc^R) transconjugants were examined on agarose gels. In seventy-two cases pED1039 had become monomeric, whereas in one transconjugant it was present as a dimer. Monomers were therefore formed in 98.75% of the mobilisation events.

Fig. 3.C

Monomerisation of dimers of pED1039 during mobilisation.

Tracks 1-5 and 7-12 show cleared lysate DNA preparation of ED3886 (pED1029, pED1039) transconjugants.

Track 6 shows a cleared lysate DNA preparation of the JC6310 (pED1029, pED1039 (dimer)) donor strain.


1 2 3 4 5 6 7 8 9 10 11 12
linear pED1029
chromosome
ccc pED1029
dimer pED1039
monomer pED1039

Warren and Clark (1980) noticed that the dimeric form of their ColE1 oriT recombinant alone sometimes formed co-integrates with ColE1 as a consequence of mobilisation. These co-integrates consisted of ColE1 and the recombinant plasmid fused at their oriT sites, and were present in 5% of transconjugants. In the experiment using JC6310 (pED1039 (dimer), pED1029) as donor, fifty-five of the seventy-three Ap^r (Spc^r) transconjugants examined also contained pED1029. In no case were pED1029 and pED1039 present as a co-integrate.

To confirm that pED1039 (dimer) did not resolve to the monomeric form merely as a result of vegetative growth, the strain was grown through approximately eighty generations, and the plasmid DNA isolated and examined with appropriate controls. All of the pED1039 DNA was still dimeric. The formation of monomers during vegetative growth was therefore very rare, if it occurred at all.

3(h) Mobilisation of R46 oriT plasmids by conjugative plasmids other than R46

oriT sequences are highly specific. Within the IncP group some plasmids have been shown not to mobilise recombinants containing the RK2 oriT sequence. This implies that there are at least two oriT sequences encoded by different IncP plasmids (Yakobson and Guiney 1983(b)). Similarly, neither F, R100 nor R1 can efficiently mobilise recombinants containing the oriT sequence of either of the other two plasmids (Willetts and Wilkins, 1984). This suggests that although these F-like plasmids encode genetically similar transfer systems, they have different oriT sequences.

The capacity of eleven IncN plasmids other than R46, to mobilise recombinants containing R46 oriT was determined (table 3.1). pED1137, pED1112 and pED1026 were used as the oriT recombinants, depending on the antibiotic resistances of the conjugative plasmid. All three oriT plasmids were mobilised efficiently by the R46 transfer system and by six other IncN plasmids, while another four IncN plasmids mobilised them at very low frequencies.

TABLE 3.1 ABILITY OF CONJUGATIVE PLASMIDS OTHER THAN R46 TO MOBILISE R46 *oriT* RECOMBINANTS

Conjugative plasmid	Inc. group of conj. plasmid	R46 *oriT* recombinant used	Transfer freq.of conj. plasmid	Mob. freq. transf.freq.
N3	IncN	pED1137	406%	1.17
pCU1	IncN	pED1112	305%	3.2×10^{-1}
269N	IncN	pED1112	70%	3.1
RM98	IncN	pED1112	405%	9×10^{-1}
R447b	IncN	pED1026	77%	1.32
893	IncN	pED1112	280%	1.75
R390	IncN	pED1112	110%	3
979	IncN	pED1026	116%	2.7×10^{-4}
pMUR274::Tn*7*	IncN	pED1137	215%	7.5×10^{-5}
pMUR545::Tn*9*	IncN	pED1137	438%	1.5×10^{-5}
825	IncN	pED1112	630%	$<6.5 \times 10^{-6}$
R388	IncW	pED1137	65%	$<3.6 \times 10^{-4}$
pUB307	IncP	pED1137	397%	3.4×10^{-5}
R446b	IncM	pED1112	121%	8×10^{-5}
pED1029	R46 Tra^+	pED1137	89%	1.8
pED1029	R46 Tra^+	pED1112	78%	2.9
pED1029	R46 Tra^+	pED1026	16%	1.9

* The transfer frequency is defined as the percentage of transconjugants which transfer the plasmid to the recipient.

Fig. 3.7

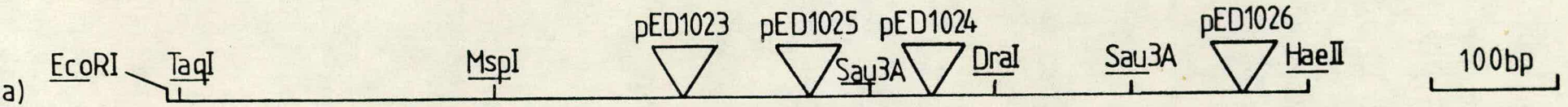
A summary diagram of the data presented in this chapter.

(a) The triangles represent Tn1725 insertions in pED939.

(c) The arrow represents the direction of transfer.

(f) The length of the arrow represents the 100bp which are thought to contain the promoter (the exact location of a sequence related to the promoter consensus sequence is suggested in chapter 4). The arrowhead represents the direction of transcription.

Fig37



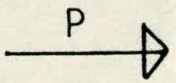
b) Region with full oriT activity

c) Region containing nick site; direction of transfer.

d) Recognition of oriT: domain 1

e) Recognition of oriT: domain 2

f) Proposed location of promoter



R446b (IncM), pUB307 (IncP) and R388 (IncW) were also screened for their ability to mobilise plasmids containing R46 oriT. These three plasmids encode rigid pili similar to those of IncN plasmids, and share their susceptibility to the pilus-specific phage PR4. However they were unable to mobilise the R46 oriT plasmids.

These results are discussed further in section 3(i).

3(i) Discussion

The mobilisation frequencies of the pED939::Tn1725 plasmids, and the recombinants derived from them (figs. 3.2, 3.3) suggested that R46 oriT has at least two parts: the site at which the DNA is nicked prior to transfer; and DNA required for recognition of oriT. The latter DNA may fulfil several functions: binding of endonuclease(s); binding of proteins involved in triggering transfer; initiation and perhaps termination of DNA synthesis; and circularisation of the plasmid after transfer. Deletion of the nick site would abolish oriT activity, whereas deletion of DNA involved in the recognition of oriT might reduce oriT activity to different degrees.

The nick site was positioned within an approximately 160bp EcoRI - Sau3A fragment (figs. 3.2, 3.3 and summarised fig 3.7). The nucleotide sequence of this region (chapter 4) revealed that this fragment was 174bp in size, and contained two pairs of inverted repeats which may form two stemloop structures (see chapter 4). These putative structures are similar to stemloops proposed to exist very near the nick site of F, and may contain the nick site of R46. The nucleotide sequence of this region also revealed a DraI target (shown in fig. 3.7) which could be used to further localise the nick site by cloning the appropriate DraI - EcoRI or Sau3A - DraI fragments (fig. 3.7).

The data described in this chapter do not rule out the possibility of a second nick site between the p.o.i. - pED1026 and the Sau3A target to its left (figs. 3.2 and 3.7). However, the oriT sequences of most plasmids are thought to contain only one nick site, such

that the nick takes place prior to transfer at a characteristic position between two specific base pairs. This appears to be the case for pSC101 (Nordheim, quoted in Willetts and Wilkins, 1984) and ColE1 (Bastia, 1978) which were isolated as relaxation complexes, and the sequence in the vicinity of the nick determined. However, evidence from nicked F oriT phages showed nicking at up to 20 different sites within a 20bp region (Thompson et al., 1984). These results are probably a consequence of packaging the nicked oriT phages, and so may not indicate that there are multiple oriT nick sites in the F plasmid.

In pED1024 and pED1025 Tn1725 had disrupted DNA required for recognition of the nick site, and so reduced the efficiency of mobilisation. These insertions, particularly pED1024 which has very low oriT activity, are comparable to a CloDF13::Tn901 plasmid isolated by Van de Pol et al. (1978). This plasmid was mobilised at very low frequencies, even when all of the CloDF13 mobilisation proteins were supplied in trans.

It is interesting that although an 8.9kb Tn1725 insertion in pED1024 and pED1025 reduced oriT activity, a 35bp remnant of Tn1725 at the same positions (in pED1032 and pED1033; fig. 3.2) did not. Presumably the 35bp insertions did not disrupt the region sufficiently to prevent recognition by the R46 transfer proteins. It is possible that the protein which recognises this region is capable of spanning 35bp to interact with DNA on both sides of the insertion, but that it is incapable of doing this for an 8.9kb insertion. The 35bp contains two 15bp inverted repeats which may form a stemloop, so reducing the length of the interuption.

The Tn1725 insertion plasmid pED1023 was mobilised by pED1029 at a frequency very similar to pED939 (fig. 3.2). However, the EcoRI deletion plasmid made from it, pED1039, was mobilised at a frequency approximately 10^{-2} fold of that of pED939 (fig. 3.2). This implicated DNA to the left of the p.o.i. - pED1023 in the recognition of oriT. This suggested that DNA on both sides of the

p.o.i. - pED1023 was required for recognition of oriT, but Tn1725 in pED1023 did not affect recognition. This can be interpreted as indicating that two distinct domains are necessary for maximum oriT activity (fig. 3.7). Perhaps the simplest explanation for this is that domain 1 and domain 2 (fig. 3.7) are both recognised by proteins to allow maximum oriT activity, and that no interaction between these two proteins is required. Even if this is the case it is surprising that domain 1 could act in pED1023, at a distance of over 9kb from the nick site, to allow high level mobilisation. The DNA required for recognition of oriT sequences from other plasmids has not been studied in detail. However, the DNA required to recognise the origin of viral strand synthesis in M13 phage (a site at which a double stranded molecule is nicked in one strand to allow unwinding of a single strand) has been studied in detail. In this system deletion of a 40bp sequence 140bp from the origin abolished replication of the phage (Dotto et al., 1982), but the phage replicated normally if a 0.75Kb fragment of foreign DNA was inserted between the origin and this 40bp sequence (Messing et al., 1977). There is therefore a precedent for the occurrence of two domains at an origin functionally similar to oriT, and further it was possible to separate these domains with foreign DNA without affecting the activity of the origin. In the case of M13 the 40bp sequence distal to the origin was required for initiation of DNA synthesis (Dotto et al., 1982). If domain 1 at R46 oriT was also involved in efficient DNA synthesis it may explain why it can be separated from the nick site by 9kb, and still maintain high efficiency transfer.

It is surprising that pED1027 and pED1141 which have lost over 200bp required for the recognition of oriT should be mobilised, even at low frequency. How this mobilisation can occur is not clear, but a similar result has been reported for the oriT of pMB1, which is contained in pBR322. Finnegan and Sherratt (1982) made a series of deletions in the vicinity of oriT of pBR322.

Deletions extending to within 45bp of the nick site were mobilised, although at frequencies approximately 100 fold lower than pBR322.

From the direction of transfer of R46 (figs. 3.6 and 3.7) it becomes clear that the first ca.300bp to be transferred contains most of the DNA required for recognition of oriT (fig. 3.7). It is possible that proteins bound to this DNA may be transferred to the recipient, and be involved in recircularisation. This is consistent with the observation that R46 oriT plasmids are circularised in recipients which do not also inherit pED1029.

One promoter was detected within the R46 oriT region which transcribes towards the transfer genes (figs. 3.4 and 3.7). The proposed location of this promoter was deduced partly from the nucleotide sequence (chapter 4), but could be confirmed by cloning the appropriate 100bp EcoRI - Sau3A fragment (fig. 3.7) into pED101. This promoter transcribes towards the transfer genes and may be required for the expression of one or more of these genes. The possibility that transcription from this promoter could be detected approximately 700bp into the transfer region was tested in recombinant pED1143 (fig. 3.4). No transcription was detected suggesting that it is terminated within these 700bp. The genetic map of the transfer region (Winans and Walker, submitted; fig. 5.1) indicates that one complementation group, traK, is positioned entirely within this 700bp. The detected promoter may, then, transcribe traK. Transcription would not be detected in pED1143 if it was terminated at the end of traK.

No other promoters were detected within the oriT region (section 3(e);fig. 3.4), which strongly suggests that there is no transcription across the nick site in R46. This is supported by the nucleotide sequence of the region which revealed no good promoter consensus sequences which would allow transcription towards the nick site.

These data make it unlikely that R46 requires transcription across oriT to allow transfer, although this has been suggested for ColE1 (Finnegan and Sherratt, 1982) and CloDF13 (Snijders et al., 1983).

In R46 and ColE1 this is also made unlikely by the fact that exposure to rifampicin prior to mating does not inhibit transfer (see chapter 5; J. Maule and N. Willetts, unpublished).

Restriction mapping positioned the HaeII - TaqI fragment containing oriT within R46. This was located between R46 co-ordinates 14.8kb and 15.7kb (figs. 1.1 and 3.5). The co-ordinates were calculated relative to the BglII target at co-ordinate 12.95 b. The 1.2kb suggested by Winans and Walker to contain oriT was located by making deletions extending from restriction targets within Tn5 and determining which deletions could be mobilised by pKM101. In this manner they mapped oriT to within co-ordinates ca.20.2kb to ca. 21.4kb. When the R46 data is used to calculate the location of oriT in pKM101 relative to the HpaI target at co-ordinate 20.1kb, then the HaeII - TaqI fragment containing oriT maps to co-ordinates 20.72kb to 21.62kb on the pKM101 map. The data of Winans and Walker and the data described in this thesis are therefore in agreement.

The thirty transfer-deficient Tn1725 insertions into R46 described in chapter 5, and twenty-three transfer deficient Tn5 insertions into pKM101 (Winans and Walker, submitted) all map on one side of oriT (fig. 5.1). It therefore seems very likely that the location determined for oriT defines one end of the R46 transfer region. This is also true of the conjugative plasmids RP4 (Guiney and Helinski, 1976) and F,(Willetts, 1972) and the non-conjugative plasmids ColE1 (Warren and Sherratt, 1978) and RSF1010 (Derbyshire, 1983).

Comparisons of the direction of transfer of the chromosome of a lac::Tn5 strain containing F oriT, or R46 oriT plasmids, and the orientation of Tn5 fragments in these plasmids, led to the conclusion that R46 transfers in an anti-clockwise direction, as drawn (figs. 3.6 and 3.7). This effectively means that the transfer genes are transferred last, which is also the case for F and RP4.

It is striking that the oriT sequences of F, RP4 and R46 are all located at the end of the transfer genes, and the direction of transfer is the same in each case. Their transfer systems are genetically distinct, with different transfer gene products, and different oriT sequences. It is possible that there is an advantage in having the oriT sequence outside the transfer genes (or in an intergenic region) as proteins bound near oriT, or nicking at oriT might otherwise affect transcription of the transfer genes. Similarly, perhaps these three plasmids transfer in the same direction, because of a selective advantage in transferring their vegetative replication genes early. They all effectively accomplish this by transferring their transfer genes last.

Dimers of the R46 oriT containing plasmid pED1039 resolved to momomers when mobilised from a recA strain containing pED1029. 98.7% of the resulting transconjugants contained pED1039 monomers. The frequency of isolation of monomers was similar to that found with an F oriT clone (95.5%, Everett and Willetts, 1982), but higher than that found with a ColE1 oriT clone (68%, Warren and Clark, 1980). This experiment is evidence that R46 oriT is cleaved, and the plasmid recircularised during transfer.

To test if the transfer genes required to recognise oriT, and probably the oriT sequence itself, are conserved between IncN plasmids, eleven were tested to determine if they were capable of mobilising a clone containing R46 oriT. Seven could mobilise one of these clones at frequencies similar to that shown by R46 (table 3.1). However, four IncN plasmids mobilised the oriT clones at much lower frequencies (varying from 4.8×10^{-4} to $<5 \times 10^{-7}$ of their own transfer frequency). Two of these four plasmids were isolated as cryptic plasmids from strains stored before the use of antibiotics, and labelled with transposons (Datta and Hughes, 1983). Although sensitive to Ike and PR4 these plasmids must have transfer systems genetically distinct from R46. The other two IncN plasmids were R-factors. This is the first report of genetic variation in the transfer systems of IncN plasmids.

It would be interesting to know if the four plasmids which do not mobilise R46 oriT have the same oriT sequence, and the same products required to recognise it. Cloning oriT from one of the four plasmids and determining if this clone is mobilised by the other three would answer this. Which of the eleven different pKM101 transfer mutants these four plasmids will complement should also be tested. This may allow identification of the complementation groups which encode proteins required for recognition of R46 oriT. Those required for this will probably not be complemented by any of the four plasmids which do not mobilise the R46 oriT clone. A similar method was used to characterise the traYZ genes of the F plasmid: R100 complemented most of the F tra mutants, but would not recognise F oriT, and this led to the conclusion that at least one of the F specific tra genes which is not required for pilus synthesis must encode a protein which recognises oriT (Reeves and Willetts, 1974).

IncP, IncW and IncM plasmids would not efficiently mobilise the R46 oriT clone. This supports the idea that these plasmids encode different oriT sequences, and specific proteins to recognise oriT, although they all encode rigid pili.

CHAPTER FOUR

THE SEQUENCE OF A 650bp MspI - HaeII FRAGMENT WITH R46 oriT ACTIVITY

4(a) Introduction

The oriT from several plasmids has been cloned into non-transferable cloning vectors, as was described in detail for R46 oriT in chapter 3. The plasmids from which oriT has been cloned include F (Johnson et al., 1980), R1, R100 and ColIb-P9 (all quoted in Willetts and Wilkins, 1984), RK2 (Yakobson and Guiney, 1983), R6K (Shafferman and Helinski, 1983), and R46 (A. Brown, 1981; this thesis chapter 3), and the non-conjugative plasmids pMB1 (Finnegan and Sherratt, 1982), ColE1 (Warren et al., 1978), RSF1010 (K. Derbyshire, 1983) and CloDF13 (Snijders et al., 1983). The sequences of the oriT regions of the conjugative plasmids F (Thompson et al., 1984), RK2 (Yakobson and Guiney, 1983) and R6K (Shafferman and Helinski, 1983) are known, as are those of the non-conjugative plasmids pMB1 (Sutcliffe, 1978), ColE1 (Oka et al., 1979), RSF1010 (K. Derbyshire, 1983), and pSC101 (Nordheim quoted in Willetts and Wilkins, 1984). However, the direction of transfer can be related to the sequence of oriT only in the case of the F plasmid.

The 622bp EcoRI - MspI fragment which has been cloned in pBR322 to form pED1137, and which gives full R46 oriT activity has been sequenced. This relatively short fragment must be recognised by several transfer proteins, and it was hoped that the sequence may reveal features such as inverted or direct repeats which could act as protein recognition sequences. Further, the sequence was determined by sequencing from the Tn1725 insertions, which allowed the insertion points of Tn1725 to be ascertained. The sequence has been compared with other oriT sequences, and notable features of the sequence are discussed.

4(b) Sequencing strategy

The R46 oriT region was sequenced using the M13 dideoxy method of Sanger et al. (1977), see chapter 2. This technique demands that the fragments to be sequenced must be cloned into vectors derived from the F specific phage M13. It was decided to clone fragments containing DNA from the R46 oriT region from pED939::Tn1725 plasmids, making use of the EcoRI targets 15bp from the ends of Tn1725. This strategy was used for a number of reasons: (a) there is a shortage of suitable restriction targets within the R46 oriT region (see section 3(b)); (b) it would allow the exact positions of the Tn1725 insertions within the R46 oriT sequence to be determined; (c) the sequence of the 15bp of Tn1725 is known (Schöffl et al., 1981), and this would act as a control to ensure the sequencing reactions were working satisfactorily, and that the correct fragments were being sequenced. The sequencing strategy is shown in fig. 4.1 and table 4.1.

Certain fragments were much more difficult to clone in one orientation than in the other. This can be exemplified by the observations made during cloning of the EcoRI - HindIII fragment of pED1137, which contains R46 oriT (See fig. 3.3), into mp8 and mp9. These two M13 cloning vectors contain the polylinker in opposite orientations (see fig. 4.1). This allowed the EcoRI - HindIII fragment to be cloned in opposite orientations in each vector. Recombinants containing this fragment cloned into mp8 were isolated at least 1000 fold more frequently than the analogous mp9 recombinants. The latter were only isolated by transferring a large number of white plaques to nitrocellulose and probing for homology to R46 oriT. No such procedure was required to find the recombinants in mp8, as almost every white plaque contained the recombinant required. It ought to have been very easy to isolate these recombinants in both MP8 and mp9 as pED1137 contains only two EcoRI - HindIII fragments, and the one not containing oriT was cut at many positions with MspI prior to the ligation.

Fig. 4.1

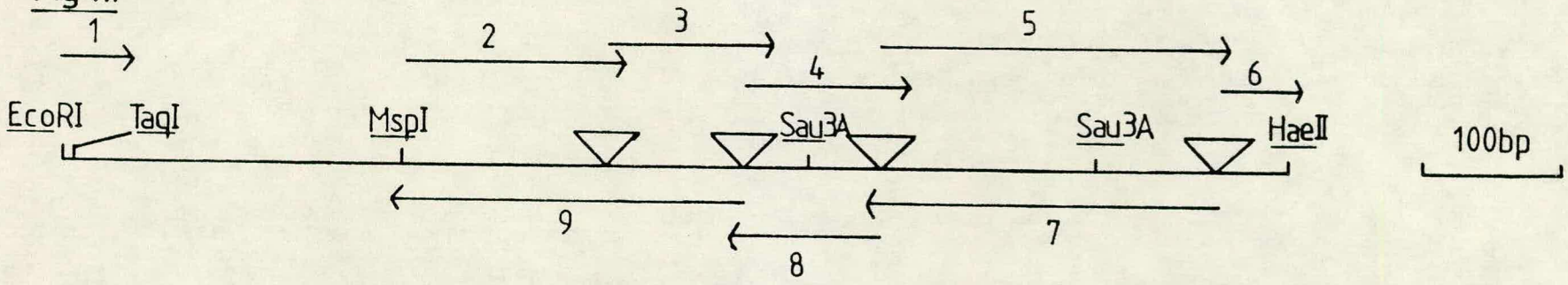
A diagram representing the sequencing strategy used to sequence R46 *oriT*.

(a) A map of the 900bp *Taq*I - *Hae*II fragment of pED939 which encode R46 *oriT*. The triangles denote the points of insertion of Tn*1725* in (from left to right) pED1023, pED1025, pED1024 and pED1026. The arrows represent the DNA sequenced from each M13 recombinant.

(b) A simple diagram of the regions used to clone fragments into M13mp8 and M13mp9, showing the positions of the *Eco*RI and *Hind*III targets relative to the primer binding site (PBS).

Fig4.1

(a)


1
2
3
4
5
6
EcoRI
TaqI
MspI
Sau3A
Sau3A
HaeII
100bp
9
8
7

(b)

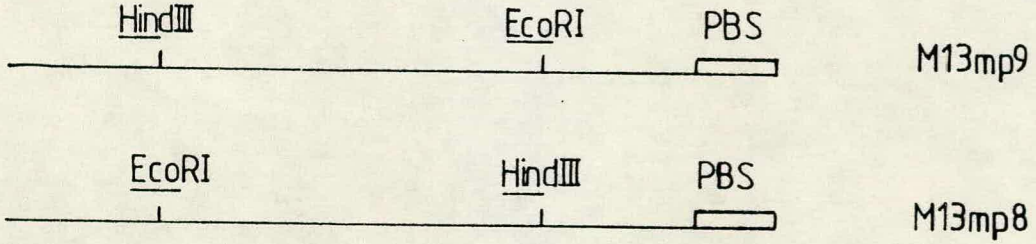

HindIII
EcoRI
PBS
M13mp9
EcoRI
HindIII
PBS
M13mp8

TABLE 4.1 SOURCE OF DNA CLONED INTO M13 CLONING VECTORS

Region Sequenced	[2]Fragment cloned	M13 vector used
[1]Arrow 1	500bp EcoRI fragment of pED1033	M13 mp9
2	622bp EcoRI-HindIII fragment of pED1137	M13mp8
3	400bp EcoRI fragment of pED1039	M13mp9
4	2.4kb EcoRI fragment of pED1033	M13mp9
5	2.3kb EcoRI fragment of pED1032	M13mp9
6	2.0kb EcoRI fragment of pED1026	M13mp9
7	622bp EcoRI-HindIII fragment of pED1137	M13mp9
8	600bp EcoRI-HindIII fragment of pED1139	M13mp9
9	500bp EcoRI-HindIII fragment of pED1138	M13mp9

1. See fig. 4.1.

2. Plasmids described in figs 3.2 and 3.3.

Overall, the three recombinants required to sequence the regions illustrated with arrows 7, 8 and 9 were very difficult to isolate. These difficulties are not understood, but may be explained by secondary structure or direct repeats within the cloned fragment interfering with M13 replication. The three rare recombinants were isolated by cloning fragments with different restriction targets at each end, and then by probing the resulting white plaques with DNA sharing homology to the 900bp oriT fragment (see above; fig. 4.1; table 4.1). However, the recombinants required to sequence the regions illustrated with the arrows 2, 3, 4, 5 and 6 were isolated very easily.

4(c) The sequence of a 650bp fragment with full R46 oriT activity

The sequence of the 650bp of the 900bp oriT fragment from the HaeII to the MspI target (see fig. 3.1) is shown in fig. 4.2. The points of insertion of Tn1725 in the four pED939::Tn1725 plasmids have been determined (fig. 4.2). The sequence of the junctions between R46 and vector DNA has been determined for the recombinants pED939, pED1137 and PED1139.

4(d) Features of the R46 oriT sequence

4(d)(i) Direct repeats

Within the sequenced 650bp, between co-ordinates 64bp and 236bp (in fig. 4.2), there are thirteen direct repeats with a consensus sequence of TGATGTA$^{A}_{C}$TTT (fig. 4.3). These fall into two groups, one of six repeats between co-ordinates 64bp and 129bp, and then after a gap of 21bp another seven repeats. The significance of these repeats is not understood, but their possible involvement in three different activities is discussed below.

Fig. 4.2

Nucleotide sequence of 650bp with full R46 oriT activity. The sequenced DNA is the EcoRI - MspI fragment cloned in pED1137 (see fig. 3.3).

The direct repeats (section 4(d)(i), fig. 4.3) are indicated with arrows.

The bases duplicated by Tn1725 insertion in, from top to bottom, pED1023, pED1025, pED1024 and pED1026 are shown in boxes. In the case of pED1023 the base pairs adjacent to only one end of Tn1725 have been determined and the first five of these bases are boxed. The duplicated bases are discussed further in section 4(e) and fig. 4.6.

The inverted repeats within the 174bp known to contain the nick site are indicated with red arrows (see section 4(d)(ii) and fig. 4.4).

The -10 and -35 sequences of the proposed promoter are shown in the red boxes.

```
TYP R460RI.SEQ;1

FROMSTADEN of:: R460RI.DAT  check: 3387  from: 1  to: 650

    FromStaden  Length: 650 bp   5-SEP-84  13:36  Check: 3387  ..

       1  CCGGCAACAT CAACTGAAGT GCCGCCCTGA TACTTTTGGG CTTCATAGTA
      51  CCCTCATTTA GAATGATGTA ATTTTGATGT ATTTCTGATG TATTTATGAT
     101  GTATTTTTGA TGTACTTTTG TTGTACTGGC TACCTCAGTA CCAGATAATG
     151  ATGTAATTCT GTTGTACTGT CAACCTTCAA GTGATGTACT TTTGATGTAA
     201  TTTTGATGTA CTTTTGTTGT AATAGTGTTG TACTGGTATT CATGCAAGCA
     251  TGAATACGGA ATATTGAACG CTTTTTAGTC AGTTAGTCCT TAACCTACGT
     301  AGATCGCTCC TACCAAAAAA GATGATTTTT TATCCCTTAA CCTGCTATAC
     351  ACCTAACGCA GCGGCATAAA TCACGCTATC ACACTGATTT AAAAAATAAA
     401  AAAATAAATA GCGTCAATAT TCGGTAATAA TTAGGTATTA AAAACACCTA
     451  ATAACTCATT GTTTTATAAT GAGTTTGCTT TATTACTTTG GTAATACTAA
     501  GATTTCAGCT TGTTAGATCC CAATCTTTTG TTTAAGAAAT GCACAATGTG
     551  AGGACTGAAT GCCAATAATA ACCGCAAAAG TATCAGATGA ACTTTTTGGC
     601  CTATATAGAC CTGGTTTCAG GAGGTAATCG GTCAGATTAT CTGCGGCGCT
$
```

Fig. 4.3

Repeat Number	Sequence	Co-ordinates
1	TGATGTAATTT	64-74bp
2	TGATGTA-TTT	75-84bp
3	TGATGTA-TTT	86-95bp
4	TGATGTA-TTT	97-106bp
5	TGATGTACTTT	108-118bp
6	TGTTGTACTGG	119-129bp
7	TGATGTAATTC	149-159bp
8	TGTTGTACTGT	160-170bp
9	TGATGTACTTT	182-192bp
10	TGATGTAATTT	193-203bp
11	TGATGTACTTT	204-214bp
12	TGTTGTAATAG	215-225bp
13	TGTTGTACTGG	226-236bp
Consensus	TGATGTA A/C TTT 13 13 9 13 13 13 13 13 9 9 13 13 13 13 13 13 13 13 13 13	

The thirteen direct repeats found within the 650bp R46 oriT sequence between co-ordinates 64bp and 236bp (fig. 4.2). A consensus sequence is shown, and the ratio below each base illustrates how often the consensus base appears at the same position in the eleven repeats.

The symbol - represents a one base pair gap introduced into the sequence to give the best alignment with the other repeats.

Firstly, although multiple direct repeats are not a feature of any of the oriT sequences so far reported (see Willetts and Wilkins, 1984), they may be involved in R46 oriT activity. This is suggested by a comparison of the oriT activities of pED1023 and pED1039 (see figs. 3.2 and 4.2) : pED1023 is fully oriT$^+$ and contains the eleven repeats, but pED1039 which has lost six repeats has an oriT activity 100 fold lower than pED1023. Furthermore, there is only 143bp present in pED1137, which has full oriT activity, but absent in pED1039. These 143bp contain six of the direct repeat sequences. Similarly, pED1028 which has lost all the direct repeats, was mobilised at a frequency 10 fold lower than pED1025 (pED939::Tn1725 from which pED1028 was derived) which contains all of them (figs. 3.2, 4.2). These data implicate the direct repeats in full oriT activity, but clearly they are not absolutely required, and they do not appear to be as important as the 92bp between the points of insertion of Tn1725 in pED1024 and pED1025 (fig. 3.2).

Secondly, these repeats may be involved in stb (a locus involved in plasmid stability) which was mapped in pKM101 by Winans and Walker (submitted) to approximately the same position. In many plasmids direct repeats are involved in two functions which affect the stability of the plasmid: incompatibility and partitioning (see Meacock and Cohen, 1980; Tolun and Helinski, 1981). These functions usually map near the origin of replication, although in IncFII plasmids the partition locus is located about 25kb from this (Miki et al., 1980; Nordström et al., 1980). In R46 the region essential for replication of R46 is approximately 11kb from the direct repeats found near R46 oriT (Brown and Willetts, 1981; Langer et al., 1981; fig. 1.1).

Thirdly, direct repeats are a common feature of replication origins and it is possible that the repeats in R46 are part of a secondary replication origin, or that they represent a remnant of a previously functional replication origin. Both of these have been found in IncF plasmids, although not near oriT. IncF plasmids contain up to three replication origins, some of which are not functional in all of these plasmids (Bergquist et al., unpublished).

4(d)(ii) Inverted repeats

Using the 'STEMLOOP' programme of the University of Wisconsin Genetics Computer Group, forty-four putative stem-loops were found within the 650bp sequence, according to the parameters described in fig. 4.4. The seven longest inverted repeats are shown in fig. 4.4.

The two inverted repeats between co-ordinates 430-475bp are within the 174bp (cloned in pED1141, fig. 3.3) thought to include the nick site. If these were to form stem loops they would form two stems of 10bp with two loops of 3bp. In one the loop would contain three adenine residues, in the other three thymidine residues (fig. 4.4). Even if these stem loops do not form the inverted repeats may act as binding sites for endonuclease(s) involved in nicking.

Inverted repeats are a feature of the oriT regions of F, RK2, ColE1, CloDF13 and RSF1010. Furthermore, the nick site of F appears to be within two pairs of inverted repeats which can be drawn as a double stemloop structure, similar to that within the 174bp which contains the R46 nick site (fig. 4.4). It would be surprising if these inverted repeats within R46 oriT are not involved with the R46 nick site, as they are the most striking feature within the 174bp known to contain the nick site, and because of their similarity with the region known to contain the F nick site.

If this structure contains the nick site, then the region from co-ordinate ca.430bp-1bp (fig. 4.2) must be transferred first. This region contains a sequence with homology to the 7bp consensus sequence of a primosome binding site. The primosome can be used to synthesise RNA primers for DNA replication. The R46 sequence is GCAGCGG (358-365bp, fig. 4.2) and the consensus sequence is GAAGCGG (Van der Ende et al., 1983).

In R46 the seven base pair sequence does not appear to be within a region of secondary structure, which makes it less likely that it could act as a primosome binding site. If R46, like F, transfers a single strand with the 5' terminus leading then the putative primosome

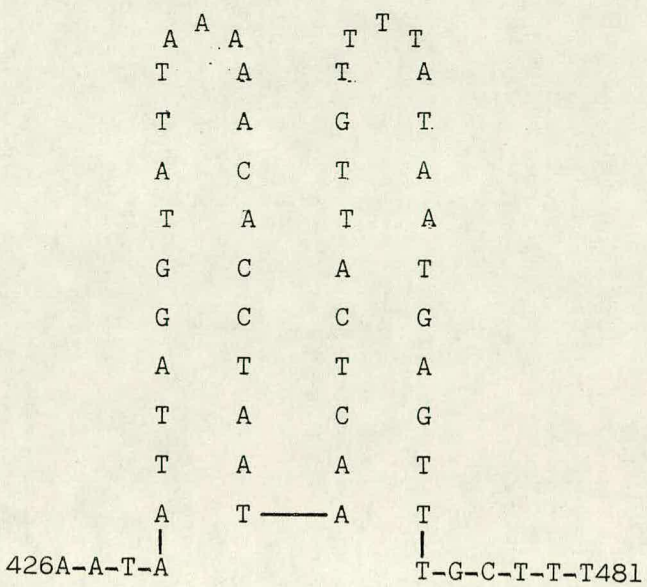
Fig. 4.4

The seven best inverted repeats found within the R46 oriT sequence. The co-ordinates correspond to those shown in fig. 4.2. The University of Wisconsin Genetic Computer Group programme 'STEMLOOP' was used with the parameters: minimum stem 6bp; minimum bonds/stem 12 (G-C = 3 bonds; A-T = 2 bonds; G-T = 1 bond); and maximum loop size 20bp.

The double stem loop structure between co-ordinates 430bp and 475bp illustrates the possible secondary structure within the 174bp which contains the R46 nick site.

Fig. 4.4

	Repeats			No. Bases Between Repeats
1.	236	GTATTCATGC	245	
				2
	257	CATAAGTACG	248	
2.	453	AACTCATTGT	462	
				3
	457	TTGAGTAATA	466	
3.	19	GTGCCGCCCTGA	30	
				4
	46	TACTTCGGGTTT	35	
4.	622	AGGTAATCGG	631	
				1
	642	TCTATTAGAC	633	
5.	430	ATTAGGTATT	439	
				3
	452	TAATCCACAA	442	
6.	121	TTGTACTGG	129	
				5
	143	ACCATGACT	135	
7.	136	CAGTACCA	143	
				18
	169	GTCATGTT	162	



binding site would remain in the donor cell. It may be required to synthesise a primer for donor conjugal DNA synthesis, if the 3' OH of the transferred strand does not act as a primer.

4(d)(iii) Promoter sequence

As shown in section 3(d) there is one promoter which transcribes away from R46 oriT, and which is located in the 245bp between the points of insertion of Tn1725 in pED1024 and pED1026. The sequence within this region which is closest to the consensus sequence of a bacterial promoter (according to Hawley and McClure, 1983) is located at co-ordinates 540-569bp (fig. 4.2). The sequence of the proposed R46 promoter (TGCACA - 18bp - AATAAT) differs in three places from the consensus sequence (TTGACA - 17bp - TATAAT). The 18bp gap between the proposed -10 and -35 sequences is close enough to the optimum 17bp to allow promoter activity (Hawley and McClure, 1983).

Fifty base pairs from this putative promoter at co-ordinates 619-624 is a sequence (AGGAGG) which could act as a very good ribosome binding site (Stormo et al., 1982), however this is not followed by a start codon at which translation could begin. It may be of interest that an inverted repeat within this region (622-642, fig. 4.4) includes three base pairs of this sequence. The HaeII target at which the sequence data ends is at co-ordinate 650bp, and presumably the translation start codon is beyond this. If this is the case then it is very unlikely that the sequence described above could act as a ribosome binding site.

4(d)(iv) An AT-rich region within R46 oriT

The 650bp region is 64.5% AT. However, the 120bp between co-ordinates 380bp and 500bp, which includes the putative secondary structure thought to be near the nick site, is 79% AT. Within this region there is a 24bp sequence (386-410bp) consisting entirely of AT. An AT-rich region has been shown to exist in the vicinity of F oriT

(Thompson et al., 1984), and to be a feature common to the vegetative replication origins of both plasmids and phages (Murotsu et al., 1981; Shafferman and Helinski, 1983; Selzer et al., 1983; Moore et al., 1978) and the bacterial chromosome (Meijer et al., 1979). AT base pairing is not as strong as GC, and so these regions may assist the separation of the complementary DNA strands which must occur at oriT and at vegetative replication origins.

4(d)(v) Homology to other oriT sequences

The 'BESTFIT' programme of the University of Wisconsin Genetic Computer group was used to search for homology between the oriT sequence of R46 and those of F,RK2, ColE1, CloDF13 and pSC101. The most interesting stretches of homology were found between the oriT of R46 and those of pSC101 and F. These are shown in fig. 4.5.

Fifteen of nineteen bases in the vicinity of the nick site of pSC101 are identical to fifteen of nineteen bases between co-ordinates 451bp and 471bp of R46 oriT. This region of R46 oriT is within one of the inverted repeats thought to be near the nick site of R46. It is not clear whether this homology is biologically significant, particularly as R46 does not mobilise pSC101 (fig. 4.5).

The homology between R46 oriT and F oriT is in the region between the points of insertion of Tn1725 in pED1024 and pED1025. This region is thought to be important for the recognition of R46 oriT. The region of homology contains a block of six adenine residues followed by six thymidine residues. This region is approximately 100 bases from the stem of the proposed double stem loop structure thought to be near the nick site of R46, and is in the region which is transferred early. In F the homologous region is ca.180bp from the nick site, but is in the region transferred last. It is, therefore, unlikely that the homologous sequences have a common function.

Fig. 4.5

R46	Sequence	451	ATAACTCATTGTTTTATAATG	471
pSC101	Sequence	1	ATAACTCTTTTATTTATCNNG	21
R46	Sequence	315	AAAAAAGATGATTTTTTAT	333
F	Sequence	302	AAAAAACATTATTTTATAT	320

Regions of homology between the R46 oriT sequence and the oriT sequence of pSC101 and F. The R46 co-ordinates correspond to those shown in fig. 4.2. The pSC101 and F co-ordinates correspond to those used by Willetts and Wilkins (1984).

Use of the University of Wisconsin Genetic Computer Group programme 'SHUFFLE' indicated that this homology was not simply due to the high AT ratio of the oriT sequences.

4 (e) Sites of Tn*1725* insertions

The sequences of the DNA adjacent to each inverted repeat of Tn*1725* in pED1024, pED1025 and pED1026 have been determined. This confirmed that in each case Tn*1725* had not deleted neighbouring DNA. This was an important observation as transposons will delete adjacent DNA, and such deletions may have been responsible for the reduction in *oriT* activity shown by pED1024 and pED1025.

Tn*1725* was derived from the minor transposon of Tn*1721* (Altenbuchner *et al.*, 1983) by the insertion of a Cm^R *Hin*dIII fragment. Tn*1721*, in common with all other Tn*3*-like transposons, has been shown to replicate 5bp of target DNA on insertion (Shöffl *et al.*, 1981; Heffron, 1983). Sequencing from these Tn*1725* insertions was expected to reveal direct repeats of 5bp of target DNA adjacent to each inverted repeat of the transposon. It was surprising to find that the insertion in pED1024 and pED1025 had duplicated 6bp and 4bp respectively. However, Tn*1725* had duplicated the expected 5bp on insertion in pED1025 (fig. 4.6 and 4.7). This heterogeneity in the number of base pairs duplicated has not previously been reported for a Tn*3*-like transposon. Only two bacterial transposable elements have been shown to duplicate a variable number of base pairs on insertion: IS*1* (8bp and 9bp; Kleckner, 1981; Iida *et al.*, 1981(a); Kanazawa *et al.*, 1984) and IS*4* (11bp and 12bp; Klaer *et al.*, 1981).

4(f) Summary of the sequences required for R46 *oriT* activity

It was shown in chapter 3 that the 558bp between the *Msp*I target and the point of insertion of Tn*1725* in pED1026 contains all the DNA required for *oriT* activity (fig. 3.3). The first 251bp contains 13 direct repeats and 4 inverted repeats (see figs. 4.3 and 4.4). Deletion of this whole region lowered the mobilisation frequency approximately fifteen fold, implying that this region may be involved in high frequency mobilisation, but that it is not absolutely required for *oriT* activity. Alternative functions for these direct repeats are discussed in section 4(d)(i).

Fig. 4.6

Plasmid	Sequence of 12bp at point of insertion	Sequence adjacent to Tn1725	Sequence adjacent to Tn1725	Bases duplicated
pED1025	5'GCATGAATACGG3' 3'CGTACTTATGCC5'	5'TCCCCTATTCA3'	5'CCCCCGAATAC3'	5
pED1024	5'ACCTGCTATACA3' 3'TGGACGATATGT5'	5'TCCCCTATAGCA3'	5'CCCCCGCTATAC3'	6
pED1026	5'TCAGATGAACTT3' 3'AGTCTACTTGAA5'	5'CCCCCATCTG3'	5'TCCCCAGATG3'	4

The R46 sequence present at the site of insertion of Tn1725 in pED939 before the insertion of Tn1725, and the sequences adjacent to Tn1725 in pED1024, pED1025 and pED1026.

The duplicated bases are underlined. The 5 bases present at the ends of Tn1725 are 5'TCCCC3' and 5'CCCCC3'.

Fig. 4.A

Sequencing gels showing sequence ladders of M13mp8 and M13mp9 recombinants.

Tracks 1-4 correspond to arrow 4 (fig. 4.1)
Tracks 5-8 correspond to arrow 9
Tracks 9-12 correspond to arrow 8
Tracks 13-16 correspond to arrow 6
Tracks 17-20 correspond to arrow 3
Tracks 21-24 correspond to arrow 7
Tracks 25-28 correspond to arrow 2

In all cases the ladders are in the order GATC.

Base pairs duplicated by Tn1725

pED1025

CATAAGCCCCC - Tn1725 - AGGGGATAAGT

The 5 duplicated bases are underlined. See bottom of tracks 1-8.

pED1024

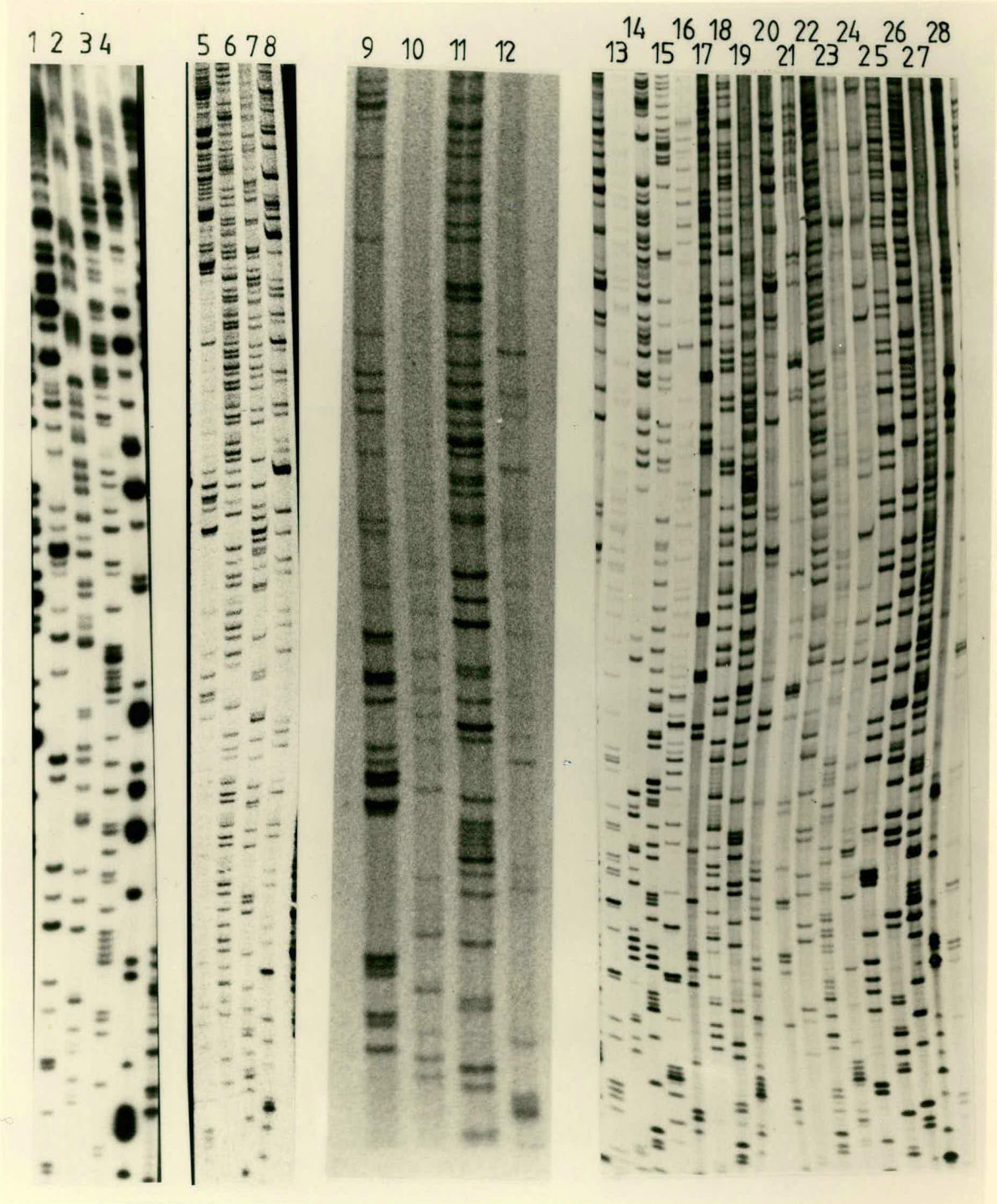
GACGATATCCCCTC - Tn1725 - GGGGGCGATATC

The 6 duplicated bases are underlined. See bottom of tracks 9-12 and 17-20.

pED1026

AGTCTACCCCC - Tn1725 - GAGGGGTCTAC

The 4 duplicated bases are underlined. See bottom of tracks 13-16 and 21-24.


1 2 3 4
5 6 7 8
9 10 11 12
13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28

The 92bp between the points of insertion of Tn*1725* in pED1024 and pED1025 must be important for *oriT* activity as pED1028 was mobilised by pED1029 at a frequency 100 fold higher than pED1027 (fig. 3.2). These 92bp are cleaved with *Sau*3A 52bp from the p.o.i. - pED1025 and 40bp from the p.o.i.-pED1024 (figs. 3.2., 4.2). This *Sau*3A target was used to clone the 215bp *Sau*3A fragment in pED967 (fig.3.3). This clone, then, contains 40bp of the 92bp present in pED1027, but absent in pED1028 and is mobilised only 2 fold higher than pED1028 (and at comparable frequencies to pED1141, fig. 3.2), implying that the 50bp between the *Sau*3A target and the p.o.i.-pED1025 are the most important sequences for recognition of *oriT* within the whole 92bp. These 50bp contain no striking features (fig. 4.2).

The 174bp between the p.o.i.-pED1024 and the *Sau*3A target at co-ordinates 516-519bp contains the nick site of R46. This region contains a 120bp sequence which is 79% AT, but its most striking feature is a possible stemloop structure between cc-ordinates 430bp and 475bp (figs. 4.2, 4.4). This is similar to the secondary structure proposed at other nick sites, but shares homology only with the nick site of pSC101.

CHAPTER FIVE

ISOLATION AND CHARACTERISATION OF TRANSFER DEFICIENT MUTANTS OF R46

5(a) Introduction

The genes required for conjugation are best understood in the F plasmid, and the F conjugation system has therefore been used as a model for other systems encoded by other conjugative plasmids. The mutants essential for the study of the F*tra* mutants were isolated by mutagenising strains containing F-primes (e.g. F*lac*) and then screening for plasmids unable to transfer (Achtman *et al.*, 1971; 1972). Transfer mutants unable to synthesise the pilus could be enriched for by picking strains containing F prime plasmids which were resistant to pilus specific phages (Ohtsubo *et al.*, 1970). These mutants were then placed in complementation groups. A major difficulty experienced in these complementation experiments was that two F primes would not co-exist stably in the same cell, because they are incompatible. This was overcome either by constructing transient heterozygotes containing two F-primes (Achtman *et al.*, 1972), or by constructing transfer deficient mutants in the compatible F-like IncFII plasmid R100, and using these to construct stable heterozygotes with the F prime mutants (Ohtsubo *et al.*, 1970).

The complementation groups defined by this process were then mapped within the transfer region by using deletions (Ippen - Ihler *et al.*, 1972), polar insertions of Mu (Helmuth and Achtman, 1975) or of Tn*10* (in R100; Foster and Willetts, 1979), as well as chimeric plasmids and phages containing fragments of the F*tra* region (Skurray *et al.*, 1976; Thompson and Achtman, 1978; Johnson *et al.*, 1981; McIntire and Willetts, 1980).

Approximately twenty genes have been shown to be required for transfer of the F plasmid (Willetts and Skurray, 1980; Moore *et al.*, 1982). Thirteen of these genes are required for pilus

assembly: tra A, L, E, K, B, V, W, C, U, F, Q, H, G (Willetts and Skurray, 1980; Moore et al., 1982). traA encodes pro-pilin which is processed by the traQ product to produce pilin; the single protein sub-unit found in pili (Minkley et al., 1976; Frost et. al., submitted). The other gene products are presumably required for sub-unit assembly and outgrowth/retraction mechanisms.

The products of traN and traG are required for the stabilisation of mating pairs, which is essential for transfer to occur (see chapter 1). Some mutants in traG also prevent pilus synthesis.

The traYZ gene products are involved in nicking and the traM product may be involved in triggering as was discussed in detail in chapter 1. The functions of the products of the other two genes (traI and traD) involved in conjugal DNA synthesis were also discussed in chapter 1: they are a DNA unwinding protein, and a protein possibly involved in DNA transfer through the membrane, respectively.

The traJ gene product is required for expression of most of the other transfer genes (Finnegan and Willetts, 1973). It acts to allow transcription from the promoter of the traYZ operon (see later; Gaffney et al., 1984; Mullineaux and Willetts, unpublished).

Transfer genes of other plasmids

The IncP plasmid RP4 and the IncN plasmid R46 (or pKM101) are the only plasmids, other than F-like plasmids, for which detailed genetic information on the transfer region is available.

Transfer deficient Tn7 insertions into RP4 mapped to one or other of three regions of the plasmid called Tral, Tra2, and Tra3 (Barth et al., 1978; Barth, 1979). Insertions into all of these regions prevented pilus synthesis and some insertions into Tral prevented transfer but did not affect sensitivity to pilus specific phage. This is in contrast to the F plasmid where all the transfer genes map to one region of the plasmid (Willetts and Skurray, 1980). Barth et al., (1978) placed their Tn7 insertions into 5 complementation groups using transient heterozygotes of two RP4:: Tn7 plasmids. Two of these complementation groups, one in Tral the other in Tra2 were required for pilus synthesis.

Four tra⁻ point mutants which were sensitive to pilus specific phages and two which were resistant to these phages were also mapped to the Tra1 region (Watson et al., 1980). This study made use of stable heterozygotes of RP4 and a chimeric plasmid containing the Tra1 region.

Transposon insertions into the IncN plasmid pKM101 indicated that this plasmid also contained three regions required for conjugal transfer (Winans and Walker, submitted; fig. 5.1). Tn5 insertions within two of these three regions prevented plating of the pilus-specific phage PR4. Winans and Walker characterised 28 Tra⁻ Tn5 insertions, and placed them in 11 complementation groups, seven of which were required for pilus synthesis.

Organisation of transfer genes

F and F-like plasmids encode their transfer system on a continuous portion of the molecule (see Willetts and Skurray, 1980). The F transfer system consists of three transcriptional units. There is a major operon of 32kb which contains twenty-one genes most of which have been shown to be required for pilus synthesis conjugal DNA metabolism and surface exclusion. The traI and traZ genes which are at the distal end of the operon are expressed at low level when unlinked to the major promoter of the operon, suggesting that there is another promoter which can transcribe these two genes (see Willetts and Skurray, 1980). Two other genes, traM and traJ, are positioned upstream of the large operon and have their own promoters (Helmuth and Achtman, 1975; Thompson and Taylor, 1982). The traYZ operon is positively controlled by the traJ product (Finnegan and Willetts, 1973). The evidence for whether traM expression is controlled by the traJ product is contradictory (Gaffney et al., 1984; Mullineaux and Willetts, unpublished). All three transcriptional units are transcribed from the transferred strand (Gaffney et al., 1984).

Most naturally occurring F-like plasmids transfer at very low frequencies since their transfer genes are repressed. F is unusual among these plasmids in being derepressed for transfer. Repression requires the products of two genes: *finP* and *finO* (Finnegan and Willetts, 1971; 1972). F is *finP*$^+$, but *finO*$^-$. γδ in F is near the position of *finO* in other F-like plasmids, suggesting the *finO* of F may have been inactivated by insertion of γδ (A.J. Clark, pers. comm.). The *finP* product of F will interact with the *FinO* product of a number of plasmids to repress the transfer of F. Plasmids capable of inhibiting the transfer of F are called Fin(F)$^+$ (for *f*ertility *in*hibition of the F plasmid). The FinOP complex acts on *traJ* and so indirectly prevents expression of the large operon (Finnegan and Willetts, 1973).

Some Fin(F)$^-$ plasmids such as IncIα plasmids R64 and R144 are repressed for transfer. These are assumed to encode repressors analogous to, but distinct from, those of Fin(F)$^+$ plasmids. Derepressed mutants of these plasmids, which transfer at high frequency, have been isolated (Meynell and Datta, 1967; Sasakawa and Yoshikana, 1978).

Other Fin(F)$^-$ plasmids such as R46 (IncN), RP4 (IncP) and R388 (IncW) are naturally de-repressed for transfer. Studies of the transfer regions of RP4 and R46 suggest their organisation differs fundamentally from that of F. Both plasmids devote approximately 20kb to transfer functions, which is considerably less than the 32kb used by F.

Secondly, and perhaps most significantly, it seems that the transfer cistrons of RP4 and R46 are transcribed from several promoters as transposon insertions were not polar on at least some nearby cistrons (Winans and Walker, submitted). The only transcriptional unit studied in detail from these plasmids is from the Tra1 region of RP4 and includes the gene which encodes the RP4 primase (Lanka *et al.*, 1984). In this case one RNA polymerase binding site, a proposed promoter, within the Tra1 region has been shown to

be required for the expression of five polypeptides. This represents one operon, and it is possible that the transfer regions of both RP4 and R46 are made up of a series of short operons.

The work described in this chapter was carried out to further our understanding of the genes required for transfer of R46. Forty Tra⁻ point mutants of R46 were characterised and thirty Tra⁻ Tn*1725* insertions were constructed. To allow the construction of stable heterozygotes between two plasmids both containing the R46 transfer genes a recombinant (pED1029) was constructed which contained the entire *tra* region of R46 cloned into pSC101. This plasmid was entirely stable in the presence of R46. Nine Tra⁻ point mutants of pED1029 were isolated. pED1029 was shown to encode the surface exclusion system of R46, and the relationship between R46 and the IncN plasmid pMUR274::Tn*7* was further examined by determining the specificity of their surface exclusion systems. Finally, transfer of R46 was shown not to be inhibited by prior exposure to rifampicin, ruling out the possibility that triggering of transfer or transfer *per se* requires transcription after mating pair formation.

5(b) Transfer-Deficient point mutants of R46

The properties of forty R46 *tra*⁻ point mutants are described in table 5.1. Thirty-five of these mutants were isolated previously (Wendy Smith and Neil Willetts, unpublished data; see table 5.1 and chapter 2), but were characterised during the course of this work. The remaining six mutants were isolated by picking PR4 resistant colonies (see chapter 2), which were later shown to be transfer deficient.

TABLE 5.1(a): TRANSFER DEFICIENT POINT MUTANTS OF R46

Plasmid	Phenotype Ap	Tet	Spc	PR4	Residual transfer frequency
pED1043	R	R	R	R	2×10^{-6}
pED1045	R	R	R	R	1×10^{-5}
pED1046	R	R	R	R	2×10^{-6}
pED1047	R	R	R	R	4×10^{-6}
pED1049	R	R	R	R	6.6×10^{-6}
pED1057	R	R	R	R	2×10^{-6}
pED1058	R	R	R	R	2×10^{-6}
pED1059	R	R	R	R	3×10^{-6}
pED1060	R	R	R	R	1.5×10^{-6}
pED1082	R	R	R	R	1.4×10^{-6}
pED1083	R	R	R	R	9×10^{-7}
pED1084	R	R	R	R	9×10^{-7}
pED1085	R	R	R	R	1.9×10^{-6}
pED1086	R	R	R	R	2.2×10^{-6}
pED1093	R	R	R	R	2×10^{-6}
pED1095	R	R	R	R	1.7×10^{-6}
pED1097	R	R	R	R	7.8×10^{-6}
pED1098	R	R	R	R	2.7×10^{-6}
pED1099	R	R	R	R	5.6×10^{-6}
pED1103	R	R	R	R	3×10^{-6}
pED1107	R	R	R	R	2.3×10^{-6}
pED1110	R	R	R	R	2.7×10^{-6}
pED1111	R	R	R	R	2.7×10^{-6}
pED1121	R	R	R	R	2.2×10^{-6}
pED1122	R	R	R	R	2.3×10^{-6}
pED1123	R	R	R	R	1.9×10^{-6}
pED1124	R	R	R	R	4.6×10^{-6}
pED1130	R	R	R	R	3×10^{-6}
pED1131	R	R	R	R	3.2×10^{-6}
pED1132	R	R	R	R	1.8×10^{-5}
pED1133	R	R	R	R	3×10^{-6}
pED1134	R	R	R	R	3×10^{-6}
R46	R	R	R	S	1.2×10^{-1}

The donor strain was ED8654 containing the appropriate plasmid. JC3272 was the recipient. The transfer frequency was the percentage of donors which transferred the plasmid to the recipient.

TABLE 5.1(b): TRANSFER DEFICIENT POINT MUTANTS OF R46

B

Plasmid	Phenotype Ap	Tet	Spc	PR4	Residual transfer freq.
pED1044	R	R	R	S	9.7×10^{-6}
pED1048	R	R	S	S	$< 2.5 \times 10^{-6}$
pED1056	R	R	S	S	$< 1.7 \times 10^{-6}$
pED1102	R	R	R	S	1.8×10^{-4}
pED1104	R	R	S	S	$< 3 \times 10^{-6}$
pED1105	R	R	S	S	4.8×10^{-5}
pED1106	R	R	R	S	$< 2.2 \times 10^{-6}$
pED1129	R	R	R	S	$< 3 \times 10^{-6}$
R46	R	R	R	S	1.2×10^{-1}

The donor strain was ED8654 containing the appropriate plasmid. JC3272 was the recipient. The transfer frequency was the percentage of donors which transferred the plasmid to the recipient.

5(c) Transfer-Deficient R46::Tn*1725* plasmids

To allow Tn*1725* insertions into R46 to be isolated using a conjugation assay (see below), Tn*1725* (Cm^R) was first inserted into the chromosome of the *E.coli* strain JC3272 (see chapter 2). R46 was transferred by conjugation into this strain (ED734), and ED734 (R46) used as a donor in filter matings in which ED3818 (Nal^R) acted as recipient. Cm^R (Nal^R) transconjugants were selected. These transconjugants were also Tet^R Ap^R Sul^R Spc^R and were assumed to be R46::Tn*1725* plasmids. Previous studies (Willetts and Foster, 1979; Brown, 1981) had shown that transposon insertions isolated from this type of conjugation assay can contain insertions within the genes required for conjugation, rendering the plasmid transfer deficient. To screen for such *tra*$^-$ R46::Tn*1725* plasmids 200 transconjugants were replica plate mated (see chapter 2) to ED24. Plasmids which would not transfer in these matings were those containing Tn*1725* inserted within the conjugation genes. In this way thirty transfer-deficient R46::Tn*1725* plasmids were isolated. Small scale plasmid DNA preparations were made and digested with *Bgl*II. The R46 *Bgl*II fragment in which Tn*1725* had inserted could be easily identified as that absent in *Bgl*II digests of the R46::Tn*1725* plasmids; a *Bgl*II fragment approximately 9kb larger than the missing *Bgl*II fragment was present in all of the digestions (Tn*1725* has no *Bgl*II targets). All thirty *tra*$^-$ plasmids had Tn*1725* inserted into *Bgl*II-A, -G or -F. These three fragments contain all the transfer genes of R46 (Brown and Willetts, 1981). The positions of the Tn*1725* insertions were then mapped more exactly by *Hpa*I, *Eco*RI, *Sal*I and *Kpn*I digestions (see figs. 5.1 and 5A).

All thirty insertions fall within the transfer region of R46 as shown in fig. 1.1 between R46 co-ordinates 35.5kb and 17.45kb. Twenty-nine fall within the three regions of pKM101 shown by Winans and Walker (submitted) to be essential for transfer (fig. 5.1). However, in pED1054 Tn*1725* has inserted almost 3kb outside any of the three distinct transfer regions (TRA-1, TRA-2 and TRA-3) of R46

Fig. 5.1

Transposon insertions in and near the transfer region of R46 or pKM101.

(a) The region of R46 between the BglII and PstI targets at co-ordinates 13kb and 36kb respectively. Each vertical line indicates a Tn1725 insertion, and the numbers denote the name of the plasmid containing the insertion.

pED960 is an R46::Tn5 plasmid isolated by A. Brown (1981).

(b) Tn5 insertions into the transfer region of pKM101. These data are from Winans and Walker (submitted).

The vertical lines show the positions of Tn5 insertions, and the letters denote the complementation group to which the gene inactivated by the transposon has been assigned.

No Tn5 insertions have been isolated within the hatched regions.

Stb, fip, nuc and eex (also called sfx) are not required for transfer (Winans and Walker, submitted).

nuc is described by Winans and Walker (1983).

The limits of TraI, TraII and TraIII are shown.

Fig.5.1

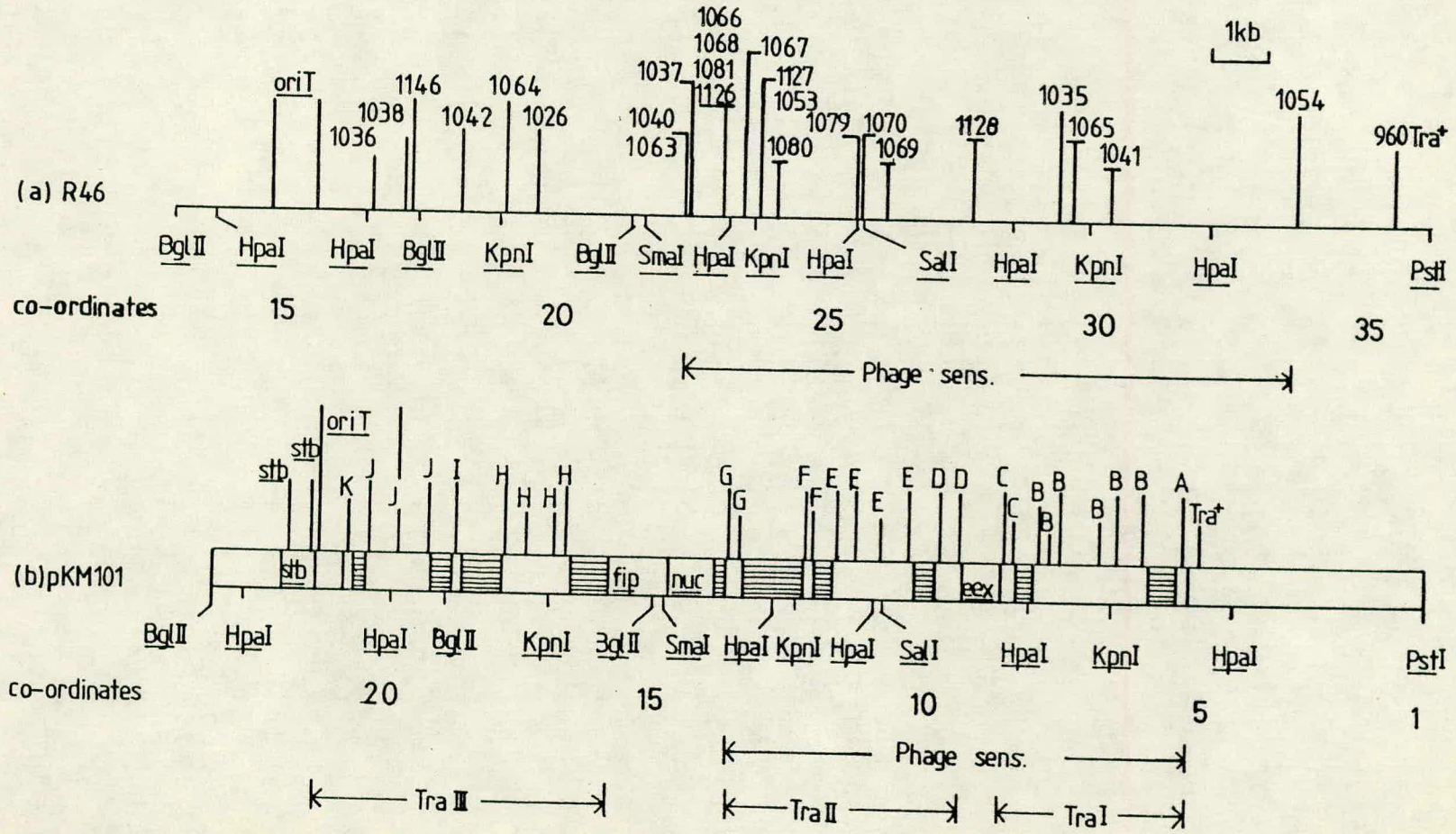

1kb
(a) R46
oriT
1146
1064
1038
1036
1042
1026
1066
1068
1067
1037
1081
1127
1126
1053
1040
1079
1070
1128
1035
1054
1063
1080
1069
1065
960 Tra+
1041
BglII HpaI HpaI BglII KpnI BglII SmaI HpaI KpnI HpaI SalI HpaI KpnI HpaI PstI
co-ordinates 15 20 25 30 35
Phage sens.
(b)pKM101
oriT
stb
stb
K
J
J
J
I
H
H
H
G
G
F
F
E
E
E
E
D
D
C
C
B
B
B
B
B
A
Tra+
stb
fip
nuc
aex
BglII HpaI HpaI BglII KpnI BglII SmaI HpaI KpnI HpaI SalI HpaI KpnI HpaI PstI
co-ordinates 20 15 10 5 1
Phage sens.
Tra III
Tra II
Tra I

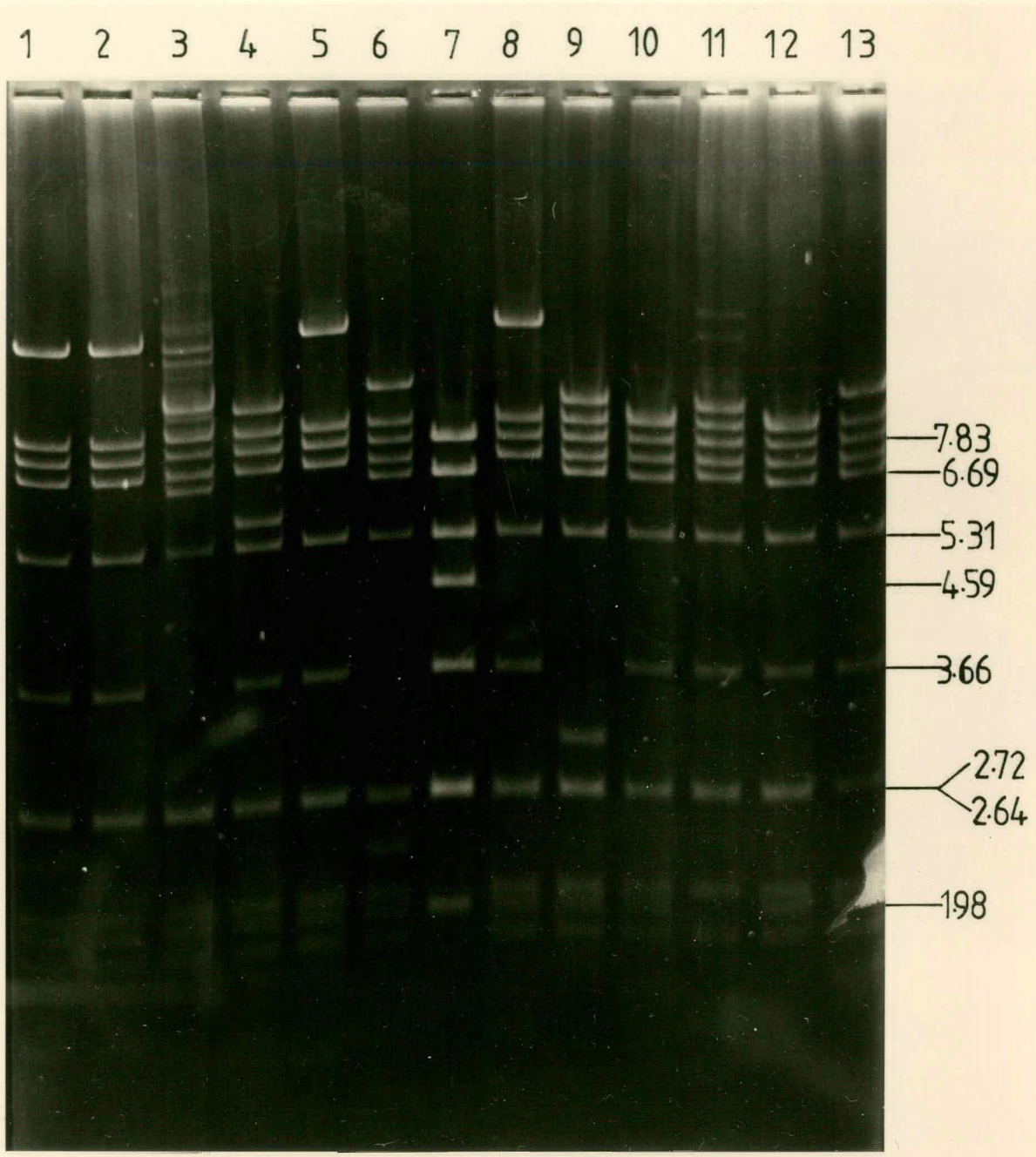
Fig. 5.A

HpaI digestions of R46::Tn1725 plasmids.

Track	DNA	Enzyme
1	R46::Tn1725	HpaI
2	R46::Tn1725	HpaI
3	pED1035	HpaI
4	pED1037	HpaI
5	pED1040	HpaI
6	pED1041	HpaI
7	pKM101	HpaI
8	pED1063	HpaI
9	pED1065	HpaI
10	pED1066	HpaI
11	pED1067	HpaI
12	pED1068	HpaI
13	pED1069	HpaI

The size standards are from the HpaI digest of pKM101 (track 7; Langer and Walker, 1981).

The map positions of most of these plasmids are shown in fig. 5.1.


1
2
3
4
5
6
7
8
9
10
11
12
13
7·83
6·69
5·31
4·59
3·66
2·72
2·64
1·98

defined by Winans and Walker. Tra^+ Tn5 insertions have been isolated in the 3kb separating Tn1725 in pED1054 from Tra1 (fig. 5.1). In pED1054 Tn1725 must have inserted in a fourth transfer region (TRA-4). Cells carrying pED1054 were resistant to PR4, so TRA-4 is probably necessary for pilus synthesis.

5(d) Cloning of the entire transfer region of R46 into pLG339

To construct stable heterozygotes between two plasmids both containing mutations in the R46 transfer region it was first necessary to clone the R46 transfer region into a plasmid compatible with R46. Brown and Willetts (1981) constructed plasmids pED935 and pED936 which are pBR325 derivatives containing a ca.23kb PstI fragment which encodes the entire transfer system of R46. The PstI fragment was cloned from pED953 (an R46::Tn5 plasmid) and extends from the PstI target at 36kb on the R46 map to a PstI target within Tn5 (inserted at co-ordinate 13.45kb).

pED935 and pED936 are compatible with R46. However, they were both lost at high frequency from cells grown without selection for the plasmid (Brown, 1981). They also have high copy numbers which would have made the *in vivo* isolation of point mutants very difficult. For these reasons the transfer region of R46 was cloned from pED935 to pLG339. The latter plasmid is a low copy number Kan^R Tet^R cloning vector derived from pSC101 (Stocker *et al.*, 1982). An EcoRI - XhoI fragment which contained the whole transfer region of R46 was cleaved from pED935. This fragment extended from the EcoRI target of pBR325 to an XhoI target within the Tn5 sequence at the other end of the DNA required for the R46 transfer functions (see fig. 5.2). The EcoRI + XhoI digest of pED935 was mixed with a SalI + EcoRI digest of pLG339 and the mixture ligated overnight (XhoI single stranded termini are complementary to those of SalI). ED8654 was transformed with the ligated mixture and the transformed cells then grown in L-broth containing kanamycin. The resulting Kan^R cells were used as donors in a mating with ED3818 (Nal^R), and

Kan^R (Nal^R) transconjugants selected. Small-scale plasmid preparations were made from the transconjugants and were cleaved with PstI or BglII. These digestions confirmed that the transconjugants contained plasmids in which the EcoRI - SalI fragment of pLG339 had been replaced by the EcoRI - XhoI fragment of pED935 containing the R46 transfer region.

This recombinant (pED1029) was shown to transfer at high frequency, and to mobilise R46 $oriT^+$ clones (see fig. 3.2). It was also sensitive to the IncN pilus specific phages PR4 and Tke.

5(e) PR4 - resistant mutants of pED1029

Eight pED1029 mutants which did not determine sensitivity to the pilus specific phage PR4 were isolated, as described in chapter 2. The ability of these strains to transfer was measured, and all were found to be transfer deficient (see table 5.2).

5(f) Surface exclusion

The presence of R46 and pKM101 derivatives in the recipient cells lowers the frequency of transfer of R46 to those cells. These plasmids therefore encode a surface exclusion system (sfx). sfx was mapped by Winans and Walker (submitted) to a position among the transfer genes (fig. 5.1), although mutants of sfx were not transfer deficient.

pED1029 should carry sfx, and in fact the presence of pED1029 in the recipient reduced the transfer frequency of R46 by 30 fold (table 5.3). Similarly, the transfer frequency of pED1029 to a strain containing R46 was reduced by a similar amount (table 5.3).

In chapter 3 four IncN plasmids were shown to encode transfer systems genetically distinct to that of R46. One of these, pMUR274::Tn7 was tested to determine if its ability to transfer was affected by the sfx of R46. As shown in table 5.3 the presence of pED1029 in the recipient did lower the transfer frequency of pMUR274::Tn7.

Fig. 5.2

The construction of pED1029.

R46 DNA is shown as a single line; pBR325 DNA as a solid line; pLG339 DNA as a double line; and Tn*5* as a hatched box.

Fig. 5.2

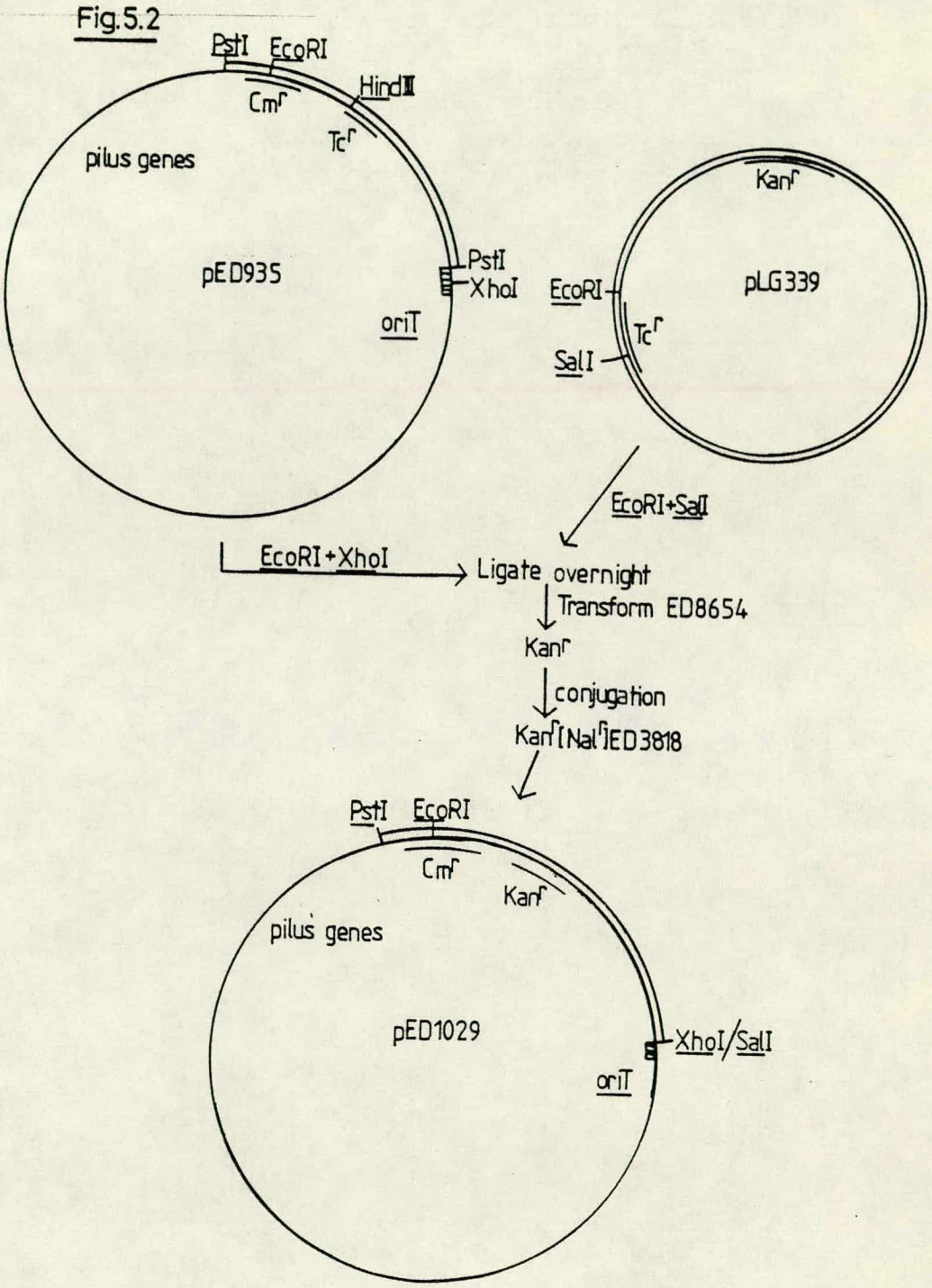

PstI
EcoRI
HindIII
Cmr
Tcr
pilus genes
pED935
PstI
XhoI
oriT
Kanr
pLG339
EcoRI
Tcr
SalI
EcoRI+SalI
EcoRI+XhoI
Ligate overnight
Transform ED8654
Kanr
conjugation
Kanr Nalr ED3818
PstI
EcoRI
Cmr
Kanr
pilus genes
pED1029
XhoI/SalI
oriT

TABLE 5.2: TRANSFER DEFICIENT POINT MUTANTS OF pED1029

Plasmid	Phenotype Kan^R	Cm^R	$PR4^R$	Residual transfer frequency
pED771	R	R	R	$<2.4 \times 10^{-6}$
pED772	R	R	R	6.4×10^{-5}
pED773	R	R	R	3.1×10^{-4}
pED775	R	R	R	3.4×10^{-6}
pED777	R	R	R	3.7×10^{-6}
pED778	R	R	R	1.3×10^{-5}
pED788	R	R	R	2.8×10^{-6}
pED792	R	R	R	3.3×10^{-6}
pED1029	R	R	S	1.5

The donor strain was ED395 containing the appropriate plasmid. JC3272 was the recipient. The transfer frequency was the percentage of donors which transferred the plasmid to the recipient.

TABLE 5.3

TRANSFERRED PLASMID	PLASMID IN RECIPIENT (ED3818)	sfx index
R46	-	1
R46	pED1029	55.8
pED1029	-	1
pED1029	R46	42
pMUR274::Tn7	-	1
pMUR274::Tn7	pED1029	88.7
pED1029	-	1
pED1029	pMUR274::Tn7	2.2

Surface exclusion indices.

An sfx index is the mating frequency with no plasmid in the recipient (ED3818) divided by the mating frequency with the appropriate plasmid present in the recipient.

TABLE 5.4

Donor		Transfer Freq.
R46/ED395	Pre-treated with Rif	27%
R46/ED395	Not treated with Rif	33%

The effect of pre-treating the donor cells with rifampicin.

However, the presence of pMUR274::Tn*7* did not reduce the transfer frequency of pED1029 (table 5.3). This implies that either pMUR274::Tn*7* does not encode an *sfx* system, or that it encodes a system with different specificity. Four surface exclusion systems have been identified amongst F-like plasmids (Willetts and Maule, 1974; see later).

5(g) Pretreatment of donors with rifampicin

The antibiotic rifampicin (rif) prevents transcription by RNA polymerase (Yarbrough *et al.*, 1976). This antibiotic was used to test whether transcription was necessary after mating pair formation to allow R46 transfer.

ED395 (R46) was grown to a cell density of approximately 2×10^8/ml, and then rifampicin was added to a final concentration of 200ug/ml. The culture was shaken for a further 30 minutes and then used as a donor to the Rif^R strain ED3822. A filter mating technique was used (see chapter 2), and the filter was incubated on an L-agar plate supplemented with rifampicin. R46 transferred at high frequency in the presence of rifampicin (table 5.4). This ruled out the necessity for expression of transfer genes after mating pair formation, and further suggested that transcription across *oriT* is not required for triggering of the transfer process (see chapter 3)

5(h) Discussion

It was originally intended to place the transfer-deficient mutants of R46 into complementation groups by constructing stable heterozygotes between them and mutants of pED1029. However, the classification of the pKM101::Tn*5* mutants of Winans and Walker (submitted) into 11 complementation groups, and the development of a method for constructing transitory heterozygotes based on transformation, reduced the priority of these experiments. It would now be possible to construct transitory heterozygotes of the transfer deficient point mutants of R46 with each of the eleven pKM101 *tra*$^-$::Tn*5* mutants which

define the eleven complementation groups. This would allow a bank of R46 tra⁻ point mutants to be classified into complementation groups. Such a bank of point mutants may be more useful than the bank of Tn5 mutants constructed by Winans and Walker because Tn5 insertions are usually polar (Berg et al., 1980). Each of the complementation groups of Winans and Walker actually represent transcriptional complementation groups which may contain more than one cistron. Some of the complementation groups are likely to have coding capacity for more than one protein, for instance traB TraE, traH and traJ are at least 2.1kb, 1.5kb, 1.3kb and 1.2kb respectively. The existence of more than one cistron within these regions might be revealed by analysis of the R46 tra⁻ point mutants.

The restriction mapping of the Tra⁻ Tn5 insertions in pKM101 allowed the limits of the transfer region to be determined (fig. 5.1(b)). Winans and Walker showed that there were two regions of 2.5kb and 0.7kb within the transfer genes which were not required for transfer since insertions into these regions did not affect transfer. This allowed the transfer region to be divided into three regions called TraI, TraII and TraIII (fig.5.1). The Tra⁻ Tn1725 insertions into R46 extend the data for pKM101. Only R46::Tn1725 plasmids which were transfer-deficient were studied, so no insertions into regions not required for transfer have been mapped. There were no Tra⁻ Tn1725 insertions within the 2.5kb and 0.7kb regions shown by Winans and Walker to be unnecessary for transfer (fig. 5.1(a)), which supports their conclusions.

The Tn1725 insertions revealed regions required for transfer which were not identified by Winans and Walker. Firstly, seven plasmids (pED1066, pED1068, pED1081, pED1126, pED1067, pED1127, pED1053) contain Tn1725 inserted within the 1.1kb region between complementation groups traG and traF. Winans and Walker were unable to show that this was required for transfer as they isolated no insertions within this region. The Tn1725 insertions confirm that it is required for transfer, and that mutations within this region

lead to PR4 resistance. It should be possible to extend the limits of traF and traG, or/and to determine whether this region contains a new complementation group by checking whether the insertions within traF and traG are complemented by any of these seven R46::Tn1725 plasmids. Secondly, pED1054, which is transfer deficient, contains Tn1725 inserted in a region not identified by Winans and Walker as necessary for transfer. This insertion is 2.3kb from the end of TraI and is separated from it by the genes KilA, KorA and KorB. The region containing Tn1725 in pED1054 will hereafter be called TraIV, and it is required for pilus formation and hence sensitivity to PR4. This must represent a twelfth complementation group required for conjugation, and an eigth essential for pilus assembly. The portion of pKM101 which must contain TraIV has not been ascribed a function by Winans and Walker, so that these results do not contradict their data. Also, TraIV is included in the fragment of DNA which encodes the R46 Tra functions in pED1029.

The organisation of the transfer genes in R46 must be very different to that in the F plasmid. As Tn5 is normally strongly polar on downstream genes it seems likely that each of the complementation groups of Winans and Walker represents a transcriptional unit encoding its own promoter. This means that there must be at least 12 transcriptional units within the R46 transfer region, and there is no evidence for a long operon comparable to the traYZ operon of F.

The ability of seven of the eleven IncN plasmids tested to mobilise R46 oriT clones suggests that the R46 transfer system is widely conserved amongst IncN plasmids (chapter 3). This has been demonstrated directly for N3 and R46 by showing that the transfer regions of these plasmids would form heteroduplexes (Brown, 1981). However, the four IncN plasmids which do not mobilise R46 oriT clones must have at least one transfer gene which is different to that of R46 (see chapter 3). The data presented in this chapter indicates that one of these plasmids (pMUR274::Tn7) does not encode a surface exclusion system which acts against the R46 transfer system.

It is not clear at this stage whether this is because this plasmid does not encode an sfx system, or whether it is because the system is of a different specificity. Four surface exclusion systems have been identified among F-like plasmids, and it has been suggested that the specificity of these is due to slight differences in the pili on the donor cell (Willetts and Maule, 1974). More experiments are required to determine if all IncN plasmids encode an sfx system and how specific these are. sfx gene(s) of pKM101 (called eex for entry exclusion by Winans and Walker, submitted) have been mapped between TraI and TraII, this implies that pMUR274::Tn7 must differ from R46 within this region.

The fact that pED1029 is excluded by R46 (table 5.3) confirms that the surface exclusion system of R46 does not operate by preventing replication of the in coming IncN plasmid, but rather that entry of the plasmid into the recipient cell is reduced. This is also the case for F-like plasmids (see Willetts and Skurray, 1980). Similarly, the surface exclusion indices shown in table 5.3 are independent of incompatibility as pED1029 and the IncN plasmids are compatible.

Pretreatment of R46 with rifampicin did not decrease the frequency of transfer of R46. R46, therefore, does not require expression of tra genes in the donor after mating pair formation. This implies that all the proteins required for transfer must be present in the cell during normal growth, and that the trigger which initiates transfer is due to a change within these proteins. This is also the case for the F plasmid (Kingsman and Willetts, 1979), ColE1, RSF1010 and IncP plasmids (Maule and Willetts, unpublished data; Derbyshire and Willetts, unpublished data).

CHAPTER SIX

CHARACTERISATION OF IS46, AN INSERTION SEQUENCE FOUND ON R46 AND N3

6(a) Introduction

Bacterial transposable elements are capable of inserting at different sites within replicons such as the bacterial chromosome, plasmids or bacteriophage DNA. They contribute a great deal of the genetic variability recently demonstrated in bacterial chromosomes and plasmids (see Iida *et al.*, 1983).

These elements fall into three classes. The first class forms two groups: insertion sequences which are small, from 768bp to 2.1kb, and encode only those functions required for their own transposition; and composite transposons which carry genes unrelated to transposition function, usually antibiotic resistance genes, flanked by two copies of the same insertion sequence which alone encodes the transposition functions. The second class are Tn3 like transposons which usually carry genes encoding resistance to antibiotics, and encode their own transposition functions. The best understood member of the third class is the bacteriophage Mu which is very complex and has a life cycle similar in part to bacteriophages, but replicates and forms lysogens via transposition. These three classes have been reviewed by Kleckner (1981).

Insertion sequences were initially detected in *E.coli* as highly polar mutations (reviewed by Starlinger and Saedler, 1976) which were shown to result from insertion of a DNA segment. The inserted sequences were short, 0.8kb- 1.5kb, and on the basis of sequence homology were of only a few different types. They were originally discovered on the *E.coli* chromosome, but were later demonstrated on plasmids and bacteriophages (Davidson *et al.*, 1975; Arber *et al.*, 1981).

The central feature of insertion sequences is their ability to integrate at sites where they were not previously present. This process is independent of DNA homology and is called transposition. Transposition typically takes place at a frequency of approximately 10^{-4} to 10^{-9} per generation (see Iida et al., 1983). In addition to transposition insertion sequences can delete or invert neighbouring DNA, can be perfectly excised, and can fuse replicons.

All of these phenomena occur in RecA$^-$ cells demonstrating that they do not take place by homologous recombination.

In addition to this non-homologous recombination insertion sequences can act as a substrate for homologous recombination. For instance the presence of two copies of an insertion sequence on a plasmid can, depending on the orientation of the copies, lead to deletion or inversion of the intervening segment of DNA after homologous recombination across the repeated sequences (see Iida et al., 1983). Another example is recombination between two copies of the same insertion sequence, one on the F plasmid and the other in the bacterial chromosome. This can lead to the integration of F into the chromosome and so to the formation of Hfr strains which transfer chromosomal markers at high frequency (Davidson et al., 1974).

Two plasmids in the same RecA$^-$ cell can fuse to form a double replicon if one of the original plasmids carries an insertion sequence. This takes place at low frequencies comparable to those of transposition. The resulting structure is called a co-integrate and has a copy of the insertion sequence at each inter-replicon boundary (Ohtsubo et al., 1981; Iida and Arber, 1980; Gill et al., 1978). These copies are always in direct repeat.

In transposition of the Tn3 like class of transposons co-integrates are usually intermediates in transposition and they are resolved by a site specific recombination system encoded by the transposon. After the resolution, which is independent of the host recombination system a copy of the transposon is present at its original position, and a second copy is present at a site in the target replicon (see Grindley, 1983).

In the insertion sequence class of transposons co-integrates do not act as intermediates in transposition. These elements transpose independently of co-integrate formation and do not encode a site specific recombination system, because when they do form co-integrates these are then stable, and are not resolved in RecA$^-$ cells (Grindley and Joyce, 1981). Transposition without co-integrate formation is called direct transposition. Co-integrate formation is thought to be closely related to transposition, and may take place by the same pathway with a co-integrate or a direct transposition event as the end product depending on the final cross-over (Galas and Chandler, 1981; Harshey and Bukhari, 1981).

Insertion sequences are often studied using their ability to form co-integrates, rather than direct transposition, as an assay. Conjugation can be used as a selection procedure for co-integrates. A recA cell containing two plasmids, only one of which is capable of transfer, can be used as a donor in a mating experiment. Transfer of the plasmid not normally capable of transfer would be selected for. This can only transfer if it is covalently attached to the transferable plasmid in the form of a co-integrate. This method has been used to study a number of different transposons (Gill et al., 1978; Guyer, 1978; Grindley and Joyce, 1981; Willetts et al., 1981). If a strain is constructed containing a plasmid known to contain no transposable elements and a second plasmid whose content of transposable elements is unknown, then using this co-integration technique the second plasmid can be screened to determine if it carries transposable elements.

Transposable elements of R46

Some evidence was previously available for the presence of insertion sequences on R46 and N3 (Brown, 1981). It had been shown that R46 contained two copies of a short sequence which was repeated in inverse orientation. This was demonstrated by cleaving R46 at its unique XhoI site, denaturing and re-annealing it, and examining it under the electron microscope. This revealed a short double

stranded region of 865bp ($\pm$30). It was also found that the restriction map of R46 constructed by Brown and Willetts (1981) differed from that of Langer and Walker (1981), and that the differences could be explained by an inversion of the DNA between the repeated sequences. This presumably occurred by homologous recombination between the repeated sequences. The restriction map of R46 suggested that each repeated sequence contained closely spaced PstI and SalI restriction targets, and that the two copies of the repeats flanked the Tc^R and As^R determinants. (Brown, 1981).

Similarly in N3 there were closely spaced PstI and SalI restriction targets on either side of the Tc^R determinant, suggesting similar repeated sequences. In N3 they were in direct orientation. Tc^S deletions of N3 had been isolated fortuitously, and preliminary restriction mapping suggested they were formed by recombination across the repeated sequences (Brown, 1981). This restriction mapping has been confirmed (Brown et al., 1984).

There was also evidence that one of the repeated sequences on R46 was capable of forming co-integrates. pED815, a Tc^R Ap^S Cm^R deletion of pBR325, was used as a target replicon for putative insertion sequences on R46 by constructing a $RecA^-$ strain containing both plasmids. This strain was used as a donor in a mating experiment, and transfer of Cm^R was selected for. This could only be transferred if pED815 was covalently attached to R46 in the form of a co-integrate. Transconjugants were isolated with the phenotype of co-integrates, and detailed restriction enzyme mapping of one of these demonstrated that it was a co-integrate. R46 was integrated into pED815 with a copy of the repeated sequence at each inter-replicon boundary (Brown, 1981).

This chapter presents evidence that both copies of the repeated sequence in both R46 and N3 form co-integrates. In each plasmid a Tc^R determinant is flanked by the insertion sequence, but this was not transposable in the form of a composite transposon. Co-integrates were shown to resolve in Rec^+ but not in Rec^- backgrounds. A simple

restriction map of the repeated sequence was constructed and Southern blots confirmed that all four sequences are homologous, as well as related to the similar insertion sequence IS15 (see below).

6(b) Formation of co-integrates containing R46 or N3 and pME420

The initial experiment of Brown (1981) used pED815, a Cm^R Tc^R Ap^S derivative of pBR325, as a target for insertion sequences present on R46. However, the Tc^R determinant of pBR325 is homologous to that of R46 (Brown and Willetts, 1981; this thesis) and this could lead to co-integration via homologous recombination, as well as to instability of co-integrates in Rec^+ backgrounds. To overcome this objection a (Cm^R Ap^R) Tc^S deletion of pBR325, pME420, which has no homology with R46 or N3 was used in these experiments. This plasmid exists in Rec^+ cells as a mixture of monomers and multimers. To overcome the problems caused by co-integrates formed between R46 or N3 and multimeric forms of pME420 it was first linearised with EcoRI, re-ligated *in vitro* and transformed directly into the *recA* strain JC6310. It was confirmed that one transformant carried a monomer, and then R46 or N3 was transferred into this strain, and the resulting transconjugants used as donors with the Rec^+ Nal^R recipient ED3818.

The strain containing R46 and pME420 gave Cm^R (Nal^R) transconjugants at a frequency of 3×10^{-5} relative to R46 transfer: all of these showed the antibiotic resistance phenotypes of both plasmids. Small scale plasmid DNA preparations were made from 15 independent transconjugants and were digested with BglII. Co-integrates formed via one or other of the repeated sequences were expected to have pME420 integrated within the BglII-A or BglII-B fragments of R46. There are no BglII targets present in pME420 so in co-integrates the BglII-A or BglII-B fragments of R46 would be replaced by a larger fragment. In nine of the isolates BglII-B was absent, whereas in the other six BglII-A was absent, and in each case a larger fragment was present. SalI digests showed that the co-integrate plasmids contained the normal complement of R46 SalI fragments, plus an extra

5.3kb fragment arising from pME420 (which has no SalI targets) flanked by two copies of the repeated sequence. This confirmed that these co-integrates had been formed via an insertion sequence present on R46, and that this insertion sequence contained a SalI target which was duplicated on the formation of the co-integrate. The insertion sequence was called IS46 and the two copies present in R46 named IS46(a) (at co-ordinate 36kb) and IS46(b) (at co-ordinate 46kb). Restriction digests of two representative co-integrates are shown in fig. 6.1, and a restriction map of R46 in fig. 1.1.

In analogous experiments, pED904 was substituted for R46. pED904 is an *in vitro* deletion mutant of R46 (Brown and Willetts, 1981), which carries IS46(a) but not IS46(b). This plasmid mobilised pME420 at a frequency of 8×10^{-6} relative to its own transfer, and BglII digests of plasmid DNA from Cm^R transconjugants showed they contained pED904::pME420 co-integrates formed via IS46(a). IS46(a) alone is therefore capable of co-integrate formation.

N3 also mobilised pME420, at a frequency of 1.5×10^{-5} of its own transfer. Plasmid DNA from 12 independent Cm^R transconjugants was examined by digestion with BglII and SalI, and all 12 carried co-integrates of N3 and pME420 formed by transposition of the sequence at co-ordinate 5kb (called IS46(d)). This copy of IS46, therefore, transposed more frequently than the one at co-ordinate 51.5kb (IS46(c)) in these experiments, although results in the following section show that IS46(c) is also capable of transposition. A restriction digest of an N3::pME420 co-integrate is shown in fig. 6.A, and a restriction map of N3 in fig. 1.1.

6(c) Formation of co-integrates containing R388 and derivatives of R46 or N3

To confirm that IS46(c) was capable of transposition the BglII-E fragment of N3, which carries IS46(c), was cloned in either orientation into the BamHI target of pBR322. The resultant plasmids had the predicted Ap^R Spc^R Tc^S phenotype, and the isolated plasmid DNA had,

as expected, one BamHI target, and a total size of 12kb. These plasmids were called pED1012 and pED1013.

R388, a Tp^R Sul^R Tra^+ IncW plasmid which has no known transposable sequences was conjugated into JC6310 containing either pED1013 or pED1012. These strains were used as donors to ED3818 selecting Ap^R (Nal^R). BamHI and SalI digestion of plasmid DNA prepared from these transconjugants showed the patterns expected for R388::pED1012 or R388::pED1013 co-integrates resulting from IS46(c) transposition. IS46(c) was therefore capable of transposition independently of IS46(d).

Although the co-integration method using R46 or N3 with pME420 provided an effective test for the presence of insertion sequences on R46 and N3, it would not detect transposition of antibiotic resistance markers independent of co-integrate formation. Since the tetracycline resistance markers in both R46 and N3 are flanked by copies of IS46, and these combinations might form composite Tc^R transposons direct transposition of these was looked for.

Transfer deficient mutants of R46 or N3 were used to look for direct transposition of the Tc^R determinants. Transfer of Tc^R from a strain containing one of the Tra^- mutants and R388, independently of the other R46 and N3 markers, would distinguish direct transposition from co-integrate formation. A transfer-deficient point mutant of R46 with residual transfer frequency of 10^{-7}, called pED899, was already available. A transfer deficient point mutant of N3 was made by isolating PR4-resistant colonies as described in chapter 2. The N3 transfer mutant was called pED1017.

A JC6310 derivative carrying R388 and pED899 transferred the pED889 Tc^R marker to ED3818 at a frequency of 10^{-6} compared to R388 transfer. Each transconjugant carried all of the pED899 antibiotic resistance markers plus Tp^R, suggesting that they carried co-integrates of the two plasmids. This was confirmed by BglII digestion of plasmid DNA from six representative transconjugants, and the fragment pattern further showed that one co-integrate had arisen via IS46(a) transposition, and five had arisen via IS46(b) transposition. Digests of two

Fig. 6.A

BglII restriction analysis of co-integrate plasmids formed via various copies of IS46.

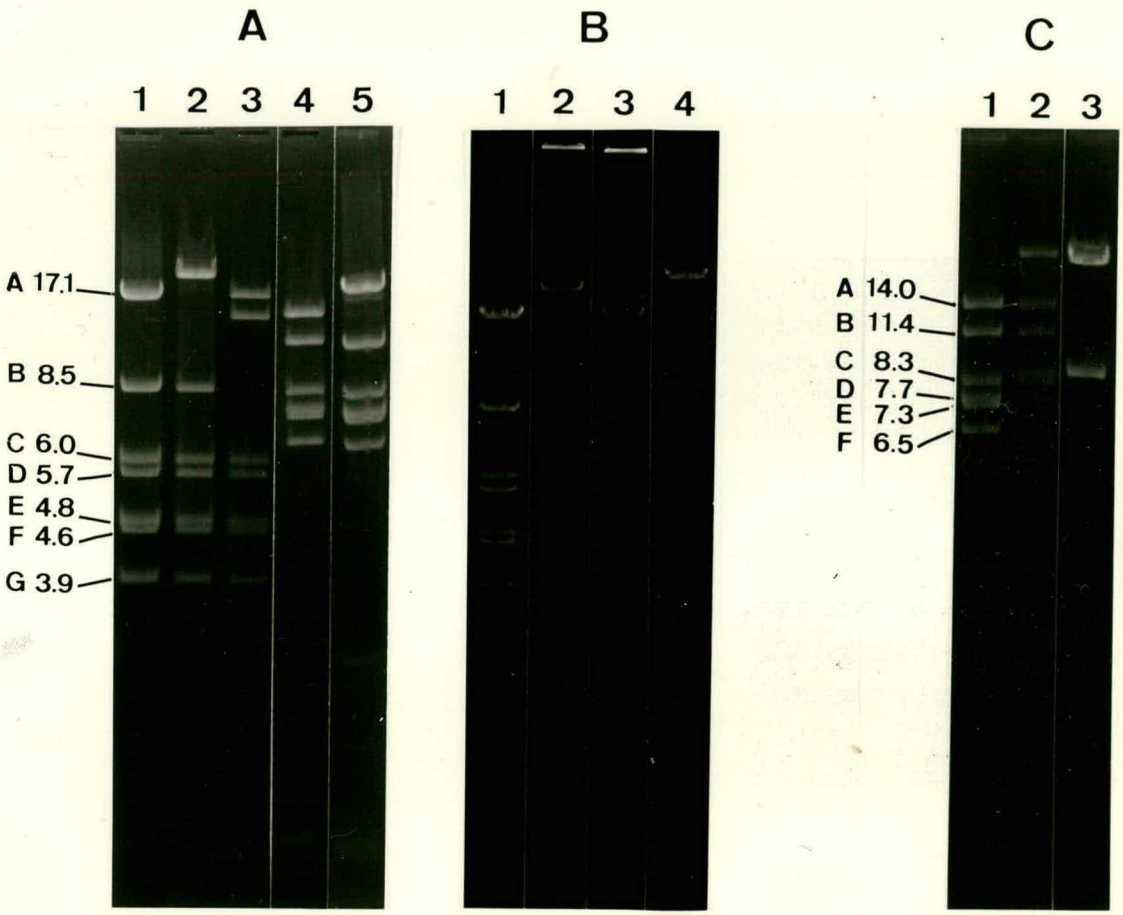
A.	Track	DNA	Enzyme
	1	R46	BglII
	2	R46::pME420 via IS46(a)	BglII
	3	R46::pME420 via IS46(b)	BglII
	4	N3	BglII
	5	N3::pME420 via IS46(d)	BglII

B.	Track	DNA	Enzyme
	1	pED889	BglII
	2	pED889::R388 via IS46(a)	BglII
	3	pED889::R388 via IS46(b)	BglII
	4	R388	BglII

C.	Track	DNA	Enzyme
	1	pED1017	BglII
	2	pED1017::R388 via IS46(c)	BglII
	3	R388	BglII

pME420 contains no BglII sites, whereas R388 has two giving fragments of 24 and 8.6kb. The sizes of the BglII fragments of R46 and pED1017 (same pattern as N3) are marked in kilobases. The fragments carrying copies of IS46 are as follows:

R46 BglII-A (IS46(a)),BglII-B(IS46(b)); N3 BglII-E (IS46(c)) and BglII-A (IS46(d)).


A
1 2 3 4 5
A 17.1
B 8.5
C 6.0
D 5.7
E 4.8
F 4.6
G 3.9
B
1 2 3 4
C
1 2 3
A 14.0
B 11.4
C 8.3
D 7.7
E 7.3
F 6.5

co-integrates are shown in fig. 6.A. Of 350 Tc^R transconjugants isolated in several mating experiments of the type described above, only 3 were Tp^R Ap^S Spc^S, the pattern expected to result from direct transposition of such a transposan. However, BglII restriction analysis showed that all three plasmids carried much larger segments of R46 DNA, and were deletion derivatives of co-integrate molecules. These data, plus the observation that none of the 21 co-integrates examined contained duplications of the entire IS46(a) - Tc^R - IS46(b) region indicate that transposition of this region occurs (if at all) considerably less frequently ($<10^{-9}$ per R388 transconjugant) than that of IS46 itself.

Similar experiments were carried out for pED1017, which had a residual transfer frequency of 5×10^{-7}. A JC6310 derivative carrying R388 and pED1017 transferred the Tc^R marker of the latter plasmid at a frequency of 9×10^{-6}, compared with R388 transfer. All of the transconjugants carried all of the pED1017 antibiotic resistance markers as well as Tp^R, but in most cases pED1017 and R388 were present as separate plasmids. This may be due to low level complementation of the transfer defect of pED1017 by R388. Clones carrying co-integrate plasmids were identified amongst the transconjugants as those which could retransfer Tc^R at high frequency in replica matings with ED24. They constituted approximately 6% of the initial Spc^R Tc^R Tp^R transconjugants. DNA from 11 of these was digested with BglII and confirmed that they were co-integrates: of these, 8 were formed via IS46(c), and 3 were formed via IS46(d). In these matings there was no evidence for the independent transposition of Tc^R, or of duplication in the co-integrates of the entire IS46(c)-Tc^R - IS46(d) region of N3. A restriction digests of a pED1017::R388 co-integrate is shown in fig. 6.A.

6(d) Resolution of R46::pME420 co-integrates

Co-integrates formed via most insertion sequences are stable in RecA$^-$ cells. They do, however, resolve at low frequency in Rec$^+$ cells to yield the original plasmid which contained the insertion sequence and the target plasmid now containing a copy of the insertion sequence. The target plasmid with the inserted insertion sequence can be used to determine the size and restriction map of the insertion sequence, as well as to study its re-transposition.

Initial attempts to resolve, in Rec$^+$ hosts, two R46::pME420 co-integrates formed via IS46(a) and IS46(b) were unsuccessful in that attempts to separate the Cm^R Tc^S pME420::IS46 resolution product by P1 transduction or by transformation failed. However, it was possible that these co-integrates had been formed by R46 insertion within the replication region of pME420, so that the pME420::IS46 resolution products would be inviable.

Digestion of R46::pME420 co-integrates with PstI yielded all the fragments of R46 plus two extra fragments. The size of these two extra fragments indicated the distance from the PstI targets in the flanking copies of IS46 to the PstI target in the Ap^R gene of pME420. The PstI digestion pattern of the two co-integrates used above suggested that R46 may indeed have inserted within the replication region, but for these co-integrates this could not be proven unequivocally.

PstI digestion of other co-integrates allowed one to be chosen (pED1030) in which R46 had integrated into pME420, via IS46(a), approximately 2kb from its PstI site. In this co-integrate R46 could not have inserted into the replication region of pME420. The PstI digest of pED1030 DNA provided preliminary evidence that resolution was occurring in the Rec$^+$ host ED3818, since visual inspection of the gels indicated that those fragments derived from the expected (high copy number) pME420::IS46(a) component were present in greater molar amounts than those derived from the (low copy number) R46 component; this had not been observed for the

other co-integrates. pED1030 was transformed from ED3818 into JC3272 and into the isogenic recA strain JC6310, and Cm^R Tc^R colonies were purified. Continued presence of the co-integrate plasmid was confirmed by showing that the antibiotic resistance markers of R46 and pME420 were transferred from these strains in filter matings at similar frequencies, and that co-inheritance was 100%. Plasmid DNA was isolated from each strain after growth through approximately 15 and 100 generations and used to transform ED8654. Cm^R transformants were selected and tested for co-inheritance of Tc^R (see Table 6.1). When the plasmid DNA from the Rec^- strain was isolated after 15 generations 20% of the Cm^R transformants were Tc^S, but this proportion remained constant after 100 generations. For the Rec^+ strain 35% of the transformants were Tc^S after 15 generations, and this proportion increased to 100% after 100 generations (table 6.1). This implied that resolution took place during the growth period in Rec^+ cells, but not in $RecA^-$. This suggested that IS46 does not encode a site specific recombination system analagous to that of $\gamma\delta$.

The isolation of small numbers of Cm^R Tc^S transformants after 15 and 100 generations growth in $RecA^-$ cells has not been explained. However, as pED1030 was originally isolated in the Rec^+ strain ED3818 resolution would have taken place in this strain. It is possible, therefore, that JC6310 was transformed with both pED1030 and the Cm^R Tc^S resolution product. This may explain why low numbers of Cm^R Tc^S transformants were found when DNA from the JC6310 strain was used to transform ED8654. To overcome this IS46 co-integrates originally isolated in a $RecA^-$ strain were used in studies of resolution and confirmed that IS46 mediated co-integrates do not resolve in $RecA^-$ strains (section 6e).

The plasmid DNA from 5 of the Cm^R Tet^S transformants was restricted with PstI and SalI which confirmed they were pME420::IS46(a) plasmids. One of these was called pED1022.

TABLE 6.1: RESOLUTION OF pED1030 IN Rec^+ AND Rec^- CELLS

Strain	Number of generations	[1]Number of Cm^R transformants/ml	[2]% of Cm^R Tet^S transformants
pED1030/JC6310	15	2.6×10^2	19%
pED1030/JC6310	100	1×10^3	20%
pED1030/JC3272	15	3×10^2	35%
pED1030/JC3272	100	1×10^4	100%

1. Plasmid DNA was isolated in small-scale preparations and used to transform ED8654. 0.1ml was spread on nutrient Cm plates.

2. 100 Cm^R transformants were patched and replica plated onto Cm nutrient plates and Tc nutrient plates.

Co-integrates are Cm^R Tet^R, resolution products Cm^R Tet^S.

The transformation technique for isolating resolution products, described above, has the advantage that the number of Cm^R Tc^S transformants is increased relative to the number of Cm^R Tc^R ones by the multicopy nature and small size of the pME420:: IS46(a) resolution product. This explains why 100% of the Cm^R transformants isolated after 100 generations of growth in the Rec^+ JC3272 strain were resolution products (see table 6.1). This technique has the disadvantage, however, that the rate of resolution cannot be meaningfully quantitated, as the transformation step effectively acts as an enrichment for resolution products.

6(e) Re-transposition of IS46(a) from pED1022

pED1022 was transformed into JC6310 (R388) to study the re-transposition of IS46(a) via co-integrate formation. In conjugation experiments between the strain and the recA recipient ED3886, the Cm^R marker of pED1022 was transferred at 10^{-6} of the R388 transfer frequency. Plasmid DNA was prepared from four independent Cm^R Tp^R transconjugants, and digestion with SalI confirmed that in each case it gave the pattern expected for R388::pED1022 co-integrates with a copy of IS46(a) at each boundary.

IS46(a) was therefore retransposable from pED1022, demonstrating that no genes required for its transposition are located elsewhere on the R46 plasmid from which it was originally derived.

Two of the pED1022::R388 co-integrates which were isolated in the recA strain ED3886 were used in resolution experiments similar to those described in section 3(d). The pED1022::R388 co-integrates were transferred in conjugation to JC3272 and JC6310 and grown through approximately 100 generations. The plasmid DNA was isolated and transformed to ED8654 selecting for Cm^R. When the co-integrates were grown in the Rec^+ strain JC3272 44% and 46% of the Cm^R transformants were Cm^R Tp^S, whereas after the same growth period in the $RecA^-$ JC6310 neither co-integrate yielded any Cm^R Tp^S transformants. This confirmed the conclusion of section 3(d) that IS46 co-integrates are very stable in $RecA^-$ cells and probably only resolve in Rec^+ cells via homologous recombination.

6(f) Physical characterisation of IS*46*

pML31, a Kan^R mini-F plasmid (Timmis *et al.*, 1978), was used by Brown (1981) as a target for transposons in N3, using conjugation to select co-integrates by the method described earlier. In this way a plasmid was isolated with the phenotype of a pML31::N3 co-integrate. P1 was then grown on a Rec^+ strain carrying this co-integrate and a Kan^R Spc^S Tc^S plasmid, called pED994, was isolated by transduction. pED994 was assumed to be a resolution product. Preliminary restriction analysis of this plasmid showed it was larger than pML31 (Brown, 1981).

*Bgl*II digestion of the pML31::N3 co-integrate has demonstrated that it was formed via IS*46*(d). Comparison of the sizes of restriction fragments in pML31 and pED994 was used to construct a restriction map of IS*46*(d). It was shown to have inserted within the 3.7kb *Eco*RI - *Hind*III fragment of pML31 so that the equivalent fragment of pED994 was 4.51kb. This indicated that IS*46*(d) was 0.81kb in length. The orientation within the *Eco*RI - *Hind*III fragment was determined from double digests using *Eco*RI together with *Sal*I or *Pst*I, and the distance from the vector *Eco*RI site to the *Sal*I site within IS*46*(d) was accurately measured as 2.41kb. The size of the internal *Sal*I - *Pst*I fragment of IS*46*(d) was 195bp.

The distance from the same *Eco*RI site to the point of IS*46*(d) insertion was then determined by electron microscopy. Heteroduplex molecules formed between *Eco*RI digested pED994 and pML31 DNA showed a single insertion loop corresponding to IS*46*(d) located at 1.94 ± 0.04kb (mean of 11 molecules) from the *Eco*RI site (see fig. 6.B). The distance from one terminus of IS*46*(d) to the internal *Sal*I site is consequently 2.41 - 1.94 = 0.47kb, and from the internal *Pst*I site to the other terminus is 810-470-195 = 145bp. The size of the single stranded loop in the heteroduplexes was measured as 0.78 ± 0.02kb (mean of 10 molecules), which is in good agreement with the size of IS*46*(d) determined from restriction enzyme digests. No double stranded "stem" bounding this loop was visible, which suggested that if inverted repeat sequences are present at the

Fig. 6.1

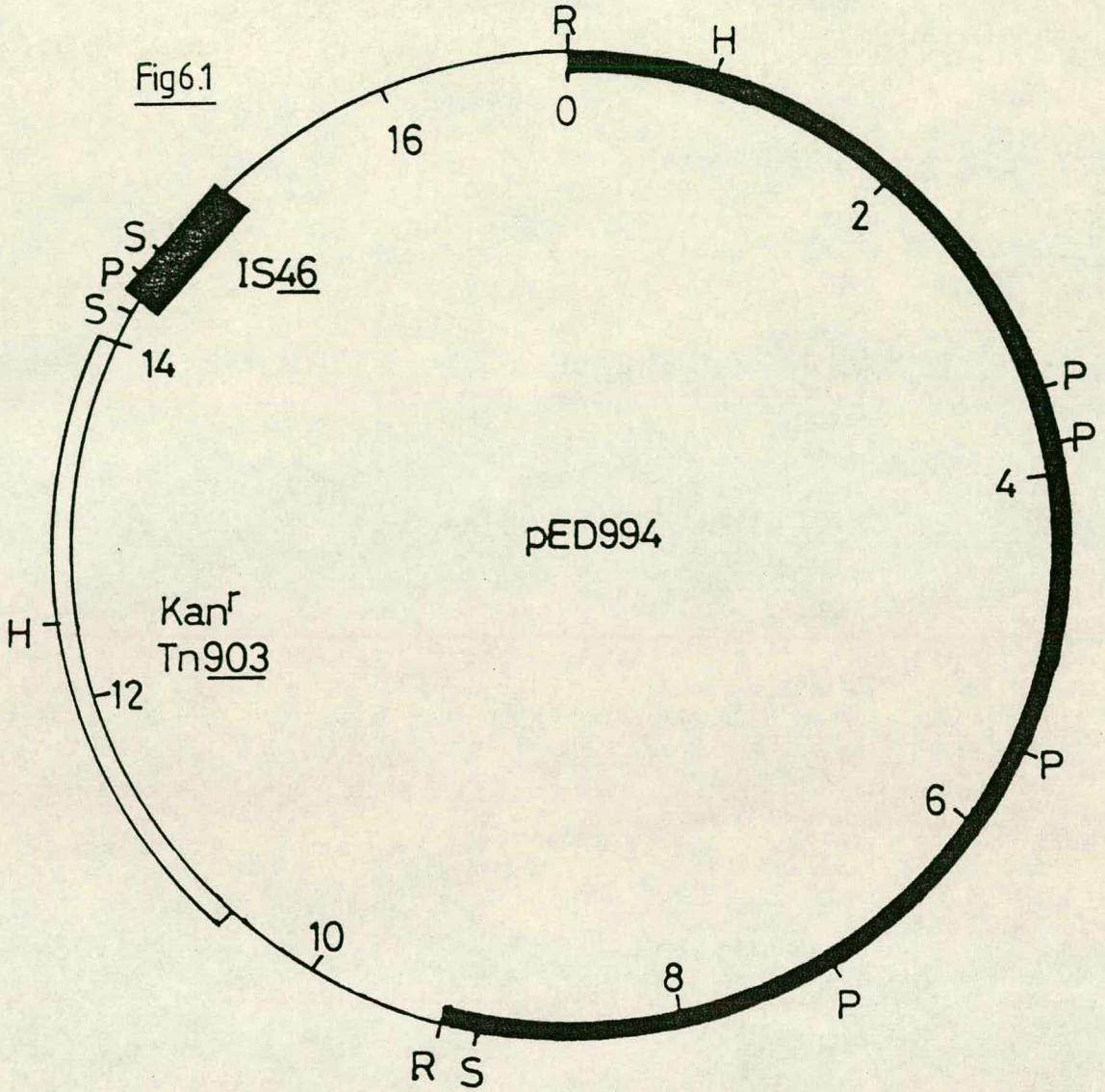
A map of pED994, a pML31 plasmid carrying an insertion of IS46(d). pM131 was constructed by Timmis et al. (1978).

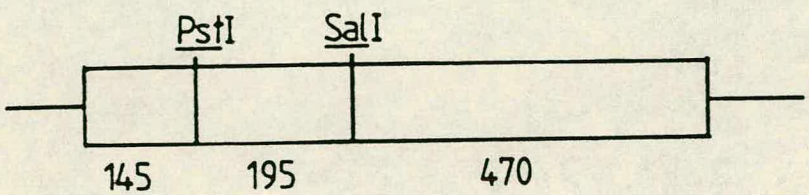
The restriction enzyme cleavage sites shown are EcoRI (E), PstI (P), SalI (S), and HindIII (H).

IS46 is shown as a solid box, the EcoRI fragment derived from F plasmid is a thicker line, and the transposon Tn903 as double lines.

A restriction map of IS46(d) is also shown. The figures denote the distance in base pairs between the restriction targets, and from these to the ends of the element.

Fig6.1


R
H
0
16
2
S
P
S
IS46
14
P
P
4
pED994
Kan^r
Tn903
H
12
P
6
10
8
P
R
S


PstI
SalI
145
195
470

IS46(d)

termini of IS46, they are probably shorter than 40bp. Maps of IS46(d) and pED994 are shown in fig. 6.1.

6(g) The four copies of IS46 show homology to each other and to IS15 but not to IS1

To demonstrate that the four copies of IS46 in R46 and N3 are homologous, the two plasmids were digested with BglII, and the fragments were separated on agarose gels and transferred to nitrocellulose filters. Duplicate filters were then hybridised to nick-translated ^{32}P-labelled pED994 or pML31 (control) probe DNA. pED994 hybridised equally strongly to each of the four BglII fragments carrying separately IS46(a), IS46(b), IS46(c) and IS46(d), thus confirming the close similarity of all four repeated sequences. pML31 did not hybridise (fig. 6.B).

Further hybridisation experiments were carried out to demonstrate the nonhomology of IS46 with IS1, an insertion sequence of similar size. First pED994 did not hybridise to the EcoRI fragment of VA λ3 (EDλ4exo::IS1) that includes IS1 (fig. 6.B).

Second, ^{32}P-labelled pBR322::IS1 DNA did not hybridise to three of the four BglII fragments of R46 and N3 which carry copies of IS46, although it did to the fourth. In a control experiment ^{32}P-labelled pBR322 DNA also hybridised strongly to the fourth fragment, BglII-B of R46; this fragment contains the Tc^{R} determinant of R46, known to be closely related to that of pBR322 (Brown and Willetts 1981). Strong hybridisation of pBR322::IS1 to the appropriate EcoRI fragment of VAλ3 confirmed that IS1 hybridisation could be detected in this experiment. It was therefore concluded that IS46 and IS1 are not closely related.

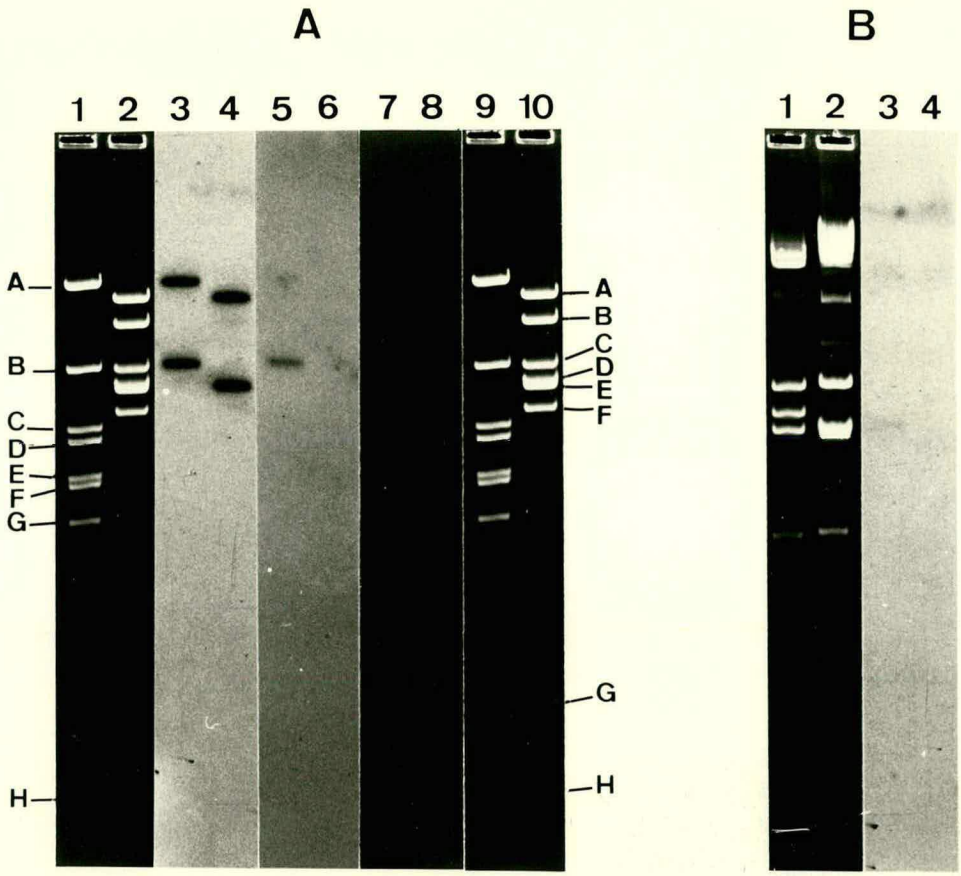
Recently, a new insertion sequence, IS15Δ, has been described that is similar in size to IS1 but does not share DNA homology with it, (Labigne-Roussel et al., 1981, 1983). Since, like IS46 this has single PstI and SalI targets ^{32}P-labelled pBR322::IS15 (called pIP1091), was used as a probe against BglII digested R46

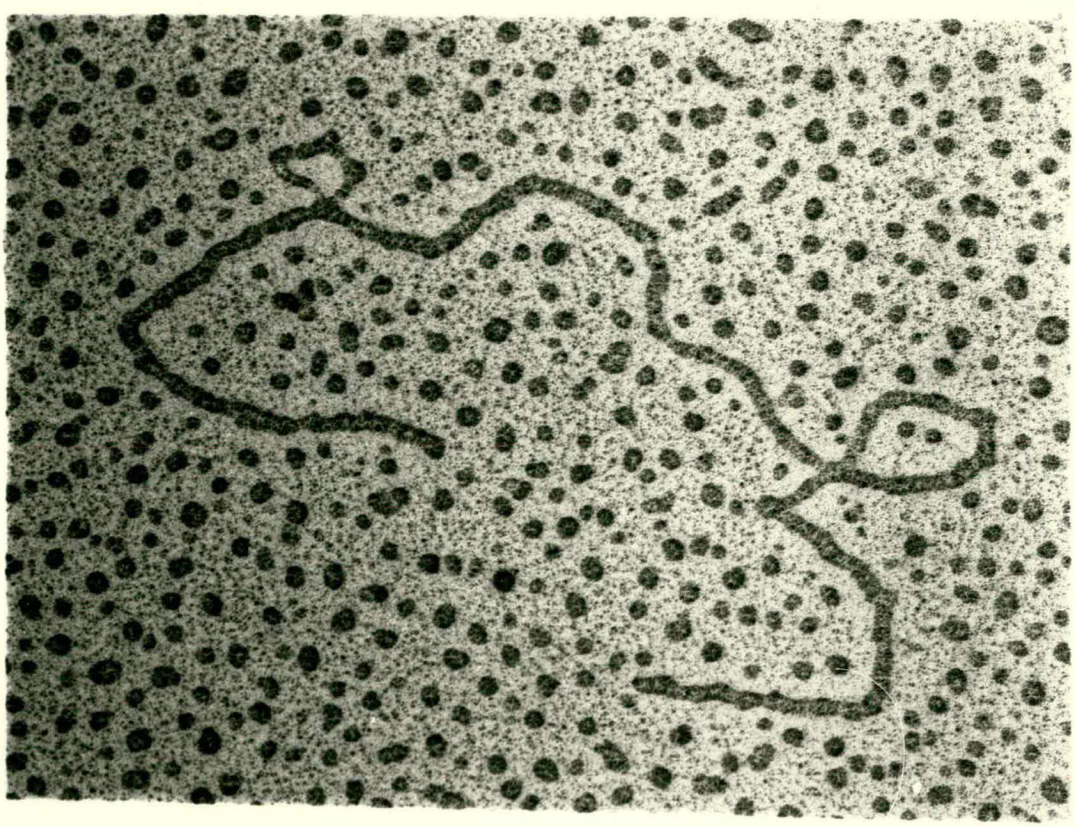
Fig. 6.B

(A) Gel photographs of the BglII cleavage fragments of R46 (lanes 1 to 9) and N3 (lanes 2 to 10) and autoradiographs of the same two lanes after southern transfer and hybridisation with ^{32}P-labelled pED994 (lanes 3 and 4), pML31 (lanes 5 and 6), or pIP1091 (lanes 7 and 8). The positions of the BglII restriction fragments of R46 and N3 are marked next to lanes 1 and 10 respectively. The weak hybridisation of pML31 to BglII-B was unexpected and is not understood, whereas the stronger hybridisation of pIP1091 to this fragment (lane 7) than to others containing IS46 is due to the identical Tc^R determinants of R46 and pIP1091.

(B) Gel photographs of the EcoRI fragments of EDλ4 (lane 1) and VAλ3 (lane 2) and autoradiographs after southern transfer and hybridisation with ^{32}P-labelled pED994.

A heteroduplex molecule formed between pML31 and pED994 after cleavage at their EcoRI targets. The 0.8kb single stranded loop represents the insertion of IS46(d) present in pED994.


A
B
1 2 3 4 5 6 7 8 9 10
1 2 3 4
A
B
C
D
E
F
G
H



and N3. Strong hybridisation to the BglII bands containing IS46 confirmed that IS46 and IS15 are indeed related (fig. 6.B).

6(h) A search for copies of IS46 on other plasmids and in bacterial chromosomes

Representative plasmids from six different incompatibility groups were screened for the presence of IS46 or related sequences by Southern blot hybridisation. Restriction digests of the plasmids F (IncFI), R1-19 (IncFII), R100 (IncFII), R68 (IncP), RSF1010 (IncQ), R388 (IncW), R390 (IncN), pCU1 (IncN) and R46 (IncN) were separated by electrophoresis in agarose gels and transferred to nitrocellulose filters. These were then hybridised with the internal SalI-PstI fragment of IS46(d), ^{32}P-labelled by nick translation. With the exception of the R46 control and pCU1, none of these plasmids exhibited homology with the IS46 probe fragment. pCU1 did hybridise and contains one pair of the closely spaced PstI and SalI targets characteristic of IS46 (Konarska-Kozlowska and Iyer, 1981).

Similarly nitrocellulose blots of EcoRI digested chromosomal DNA from a variety of species of the families Enterobacteriaceae and Pseudomonadaceae were hybridised with the IS46 specific probe. R46 DNA was used as a hybridisation control on the same filters. The species tested were E.coli, Salmonella typhimurium, Shigella sonnei, Proteus morganii, Serratia marcescens, Providencia stuartii, Enterobacter aerogenes and Pseudomonas aeruginosa. None of these chromosomal DNA preparations showed any significant homology with IS46.

6(i) Discussion

The work described in this chapter demonstrates that R46 and N3 each contain two insertion sequences. Restriction mapping showed that they all contained similarly spaced PstI and SalI restriction targets, and Southern blots confirmed that they share homology with each other. The insertion sequence was called IS46.

Co-integrate formation between R46 or N3 and the non-mobilisable plasmid pME420, or between R388 and transfer mutants of R46 or N3 was used as an assay for the transposition of the insertion sequences. Co-integrate formation took place in the recA strain JC6310, as expected for an insertion sequence based mechanism. Restriction enzyme digestions demonstrated that IS46 had been duplicated on the formation of the co-integrates with copies of the sequence at each inter-replicon boundary, and that it had inserted at different locations within the target plasmid. These findings are typical of co-integrate formation via insertion sequence transposition.

Restriction digests also reveal which copy of IS46 was involved in the formation of the co-integrate. Each of the four copies of IS46 transposed with approximately similar frequencies which varied from 10^{-7} to 10^{-5} according to which assay system was used. These frequencies are similar to those reported for other insertion sequences, (Iida et al., 1983). None of the other inverted repeat sequences on R46 (see fig 1.1) or N3 (Brown 1981) were found to be duplicated in any of these co-integrates, so either these are transposable at frequencies that are one or two orders of magnitude less than that of IS46 or are not transposable at all.

Resolution of pME420::R46 and R388::pED1022 co-integrates was followed in Rec^+ and $RecA^-$ strains. A transformation technique which enriched for the small, high copy number pME420::IS46 resolution product was used to detect resolution. It occurred only in the Rec^+ strain. This implied that in common with other insertion sequences and in contrast to γδ and Tn3, IS46 does not encode a site specific recombination system allowing efficient resolution of co-integrate molecules. Lack of such a system is also implied by the stability of the Tc^R determinant of N3 which is flanked by copies of IS46 in direct repeat, and would be lost at high frequency if IS46 contained a site specific resolution system. Resolution of IS46 mediated co-integrates, therefore, relies upon

reciprocal recombination between two copies of IS46, brought about by the host dependent, generalised recombination system, which is relatively inefficient for such short regions of homology (Grindley and Joyce, 1981; Berg et al., 1981).

Dodd and Bennett (1983) have recently described a resolution site (res) located on the PstI-F fragment of R46, which contains the terminal 135bp of IS46(a). They showed that approximately 11% of their TnA insertions into R46 had suffered deletions with one end-point at the res site of TnA and the other at a specific site within the PstI-F fragment of R46. Further they showed that R46 encoded on analogue of the tnpR protein of TnA. Resolution of TnA co-integrates by site specific recombination requires tnpR, and R46 was shown to complement TnA tnpR mutants. Although IS46(a) is very near the res site of R46 the resolution system of R46 is unlikely to be encoded by IS46 because: pME420::R46 co-integrates are stable in RecA$^-$ cells; pED1022::R388 co-integrates are stable in RecA$^-$ cells also containing R46; and the Tc^R determinant of N3 is stable (see above). Further, if IS46 encoded the res site one may have expected Dodd and Bennett tc have isolated deletions with end-points in IS46(b), but none were isolated. These conclusions are confirmed by the more recent observation that IS46(a) is inserted within a transposable element similar to, but distinct from, TnA and γδ. The deletions are due to site specific recombination between the res site of TnA and that of the inactivated transposon (P. Bennett, pers. comm.).

A number of well characterised transposons have insertion sequences at their termini. The transposition functions of these composite transposons are encoded entirely by one or both of the terminal insertion sequences. The insertion sequence at the ends of Tn903 (IS903 - Kan^r-IS903) are identical and both encode transposition functions (Grindley and Joyce 1981). However in Tn5 (IS50 - Kan^R - IS50) and Tn10(IS10 - Tet^R - IS10) one of the flanking insertion sequences is mutated such that it no longer encodes the functions required for transpositon. Mutated insertion sequences

can still transpose when transposition functions are supplied *in trans* (Rothstein *et al.*, 1980; Foster *et al.*, 1981). It seems likely that any sequence flanked by insertion sequences will transpose in the form of a composite transposon. This is particularly evident for IS*1* which is at the ends of a number of composite transposons: Tn*9* (MacHattie and Jackowski, 1977), Tn*1681* (So *et al.*, 1979), Tn*2350* (Clerget *et al.*, 1980) and Tn*2571* (Iida *et al.*, 1981(b)). An *in vivo* system for the formation of new Cm^R IS*1* flanked composite transposons was devised by Iida *et al.* (1981(c)). An IS*1* element was transposed to a site in the vicinity of a Cm^R determinant on a plasmid, and the Cm^R gene was then transposed onto the genome of λ. This transposition required the transposition of a second IS*1* to the other side of the Cm^R gene prior to its transposition to λ. These new composite transposons were subsequently shown to transpose from their location on λ.

Considering the data described above for other insertion sequences it seemed likely that the IS*46* - Tc^R - IS*46* regions of N3 or R46 may act as composite transposons. However, none of the R46::pME420 or N3::pME420 co-integrates analysed by *Bgl*II restriction showed duplications of the IS*46* - Tc^R - IS*46* regions, and were not formed via a composite transposon.

However, the composite transpons so far studied appear to transpose directly at a higher frequency than that of co-integrate formation (N. Grindley pers. comm.), and so direct transposition of the IS*46* - Tc^R - IS*46* regions on N3 and R46 was looked for. No such transposition from transfer deficient mutants of R46 or N3 to R388 was detected, and if generated at all must have been present at $<3 \times 10^{-9}$ per cell. These results may simply reflect the distance between the IS*46* elements in each case: Chandler *et al.* (1981) showed that for IS*1* flanked composite transposons there was a two fold decrease in the frequency of transposition for each extra kilobase of DNA between the insertion sequences. If this is also true for IS*46* then the IS*46* - Tc^R - IS*46* region ought to form co-integrates 1000 fold less often than IS*46*.

The other possibility is that IS46 is only capable of co-integrate formation and not of direct transposition (or that it forms co-integrates many fold more frequently than it transposes directly). This has been suggested for IS15Δ , which is closely related to IS46 (see below). IS15Δ was unable to transpose directly, although IS15, which is derived from IS15Δ (see below), was capable of transposing directly in the same type of experiment (Labigne-Roussell and Courvalin, 1983). Further Tn2680 which is flanked by directly repeated copies of IS26, which is closely related to IS15 and IS46, was apparently unable to transpose directly (Iida et al., 1982), while Tn1525 which is flanked by directly repeated copies of IS15 could transpose directly in a similar system (Labigne-Roussel and Courvalin, 1983).

The sequence of IS15 has been determined (Trieu-Cuot and Courvalin submitted), and has illustrated that IS15 is a copy of IS15Δ inserted into itself. The open reading frames of IS15Δ have been altered by the formation of IS15, and the authors suggest that the altered gene products may have led to a transposition merchanism based on direct transposition rather than co-integrate formation.

The physical characterisation of IS46 plus the inability of IS46 to hybridise to plasmids containing IS1 (pBR322 :: IS1, R100 and R1-19), IS2(F), IS3(F), γδ(F), IS10 (R100), IS21 (R68) or Tn1 (R1-19) showed that it is distinct from those transposable elements as well as from IS4, IS5, IS50 and IS903 (Kleckner, 1981). However, IS46 seems to be related to the insertion sequence IS15 (1744bp) and IS15Δ, IS26 (Iida et al., 1982) and IS140 (Brau and Piepersberg, 1983) all of which are approximately 800bp. These elements have been shown to contain similarly spaced SalI and PstI restriction targets.

The sequence of IS15 confirms the physical map of IS46 described in this chapter: the length of IS15Δ is 820bp compared to ca.810bp for IS46; the IS15Δ internal PstI - SalI fragment is 193bp compared to ca.195bp for IS46; from the SalI site to the end of IS15

is 457bp compared to ca.470bp for IS46. IS15 has been shown to replicate eight base pairs on insertion, and to have inverted repeats of 14 base pairs at its ends (Trieu - Cuot et al., 1983).

IS46 related elements are present on plasmids belonging to a variety of incompatibility groups including IncA (RA1), IncC (pIP1031 and R40a), IncF (pJR62 and R124; but not F, R100 or R1-19), IncI (several including pIP112, pIP565 and R144), IncM (pIP135, pIP151, pTH1), IncT (RTs1) and IncY (pIP231), as well as on the IncN plasmids R46, N3 and pCU1 (Brau and Piepersberg, 1983; Iida et al., 1982; Labigne - Roussel and Courvalin, 1983). Unexpectedly it was not present on the IncN plasmid R390, despite its similarity to other IncN plasmids. Although widely distributed on plasmids, homology to IS46 was not detected on the chromosomes of several species of enterobacteria or P.aeruginosa : this contrasts to the other small insertion sequence IS1, which although not found in Pseudomonas strains (Willetts et al., 1981) is fairly widely distributed amongst the Enterobacteriaceae (Saedler and Heiss, 1973; Nyman et al., 1981).

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Characterization of IS*46*, an Insertion Sequence Found on Two IncN Plasmids

ANTHONY M. C. BROWN,† GEORGE M. COUPLAND, AND NEIL S. WILLETTS‡*
Department of Molecular Biology, University of Edinburgh, Edinburgh EH9 3JR, Scotland

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The IncN plasmids R46 and N3 each contain two copies of an insertion sequence which we denote IS*46*. This insertion sequence has single *Pst*I and *Sal*I restriction sites and is 0.81 kilobases long. All four copies of IS*46* were capable of forming cointegrates, although the DNA between the insertion sequences, which in each case carries a tetracycline resistance gene, was not transposable in the form of a compound transposon. IS*46*-mediated cointegrates resolved in Rec$^+$ but not in RecA$^-$ cells. Recombination between two copies of IS*46*, causing an inversion, accounts for the existence of two distinct forms of R46. IS*46*-mediated deletions were probably responsible for the formation of the plasmid pKM101 from R46. IS*46* was not homologous to IS*1* but did show homology with IS*15*.

Insertion sequences form one important group of transposable DNA elements; they differ from transposons in carrying no readily detectable marker and in being usually smaller in size (for a review, see ref. 34). Insertion sequences have been found on the chromosomes of *Escherichia coli* and other enterobacteria and in plasmids either as individual elements or in pairs bounding antibiotic resistance markers, forming composite transposons.

Only a relatively small number of plasmids of various incompatibility groups have so far been screened for the presence of insertion sequences. In the course of our studies on the IncN plasmids R46 and N3 (8; A. Brown, Ph.D. thesis, University of Edinburgh, Edinburgh, Scotland, 1981), we encountered phenomena suggesting that each plasmid carries two copies of an insertion sequence. In both R46 and N3 these insertion sequences bound the Tc^r region of the plasmid in inverted and direct repeat orientations, respectively. This paper describes these experiments and others designed to characterize the sequences (called IS*46*) to show that they are interrelated and to determine their relationship to insertion sequences described previously. Also, unsuccessful attempts were made to transpose the Tc^r determinants of R46 and N3 as composite transposons.

MATERIALS AND METHODS

Bacterial strains, plasmids, and phages. The characteristics of *E. coli* K-12, *Enterobacteriaceae*, and *Pseudomonas* strains, and of their plasmids and bacteriophages, are described in Table 1.

Media and buffers. Culture media were described by Willetts and Finnegan (67), and antibiotic additions were described by Brown and Willetts (8).

Genetic techniques. Quantitative membrane filter matings were carried out as described by Brown and Willetts (8), and transformation was carried out by the technique of Lederberg and Cohen (41). P1 *vir* a transduction was by the method of Willetts et al. (66).

DNA preparation and manipulation. Plasmid DNA was prepared by a procedure based upon the cleared lysate technique of Clewell and Helinski (12) and the polyethylene glycol precipitation technique of Humphreys et al. (30). It was then purified by cesium chloride-ethidium bromide density gradient centrifugation. Small-scale plasmid preparations were made by the method of Birnboim and Doly (5).

Bacterial DNA was prepared as described by Willetts et al. (66). Large-scale preparation of lambda phage lysates and extraction of the phage DNA were described by Willetts (63).

The use of restriction enzymes for cleavage analysis and cloning was described by Brown and Willetts (8).

Southern hybridization techniques. For hybridizations, DNA fragments separated on agarose gels were denatured and transferred to nitrocellulose filters by the methods of Southern (57). The filters were rinsed in 2× SSC (1× SSC is 0.15 NaCl plus 0.015 M sodium citrate) and baked at 80°C for 2 h. Plasmid DNA was nick translated as described by Willetts et al. (66), after Rigby et al. (52). The 195-base-pair (bp) *Sal*I-*Pst*I fragment of IS*46* was also labeled by this method after extraction from an 8% acrylamide gel by the method of Maxam and Gilbert (43).

The nitrocellulose filters were presoaked for 1 h by shaking at 37°C in hybridization fluid: 4× SSC (0.6 M NaCl, 0.06 M sodium citrate), 50% formamide, 0.1% sodium dodecyl sulfate, and 1× Denhardt solution (0.02% bovine serum albumin, 0.02% polyvinylpyrrolidone, 0.02% Ficoll). Hybridization was then carried out overnight at 37°C in a plastic bag containing hybridization fluid (20 ml for a 12-lane gel) plus 500 μg of denatured salmon sperm DNA and 10^5 to 10^6 cpm of denatured probed.

After the hybridization, filters were washed twice for 1 h under hybridization conditions and twice for 1 h in 2× SSC. The filters were then dried at 37°C. Autoradiograms were usually exposed for 1 to 2 days at −70°C, using preflashed X-ray film (Dupont Cronex 4) and a phosphotungstate intensifying screen (Dupont Cronex).

Electron microscopy. Self-annealing, heteroduplexing, and formamide spreading of DNA molecules were by the method of Davis et al. (18). The molecules were spread on Parlodion grids, and φX174 double-stranded (5.38 kilobases [kb]) and M13 single-stranded (6.23 kb) DNA circles were added to provide length standards. Grids were examined, and molecules were photographed with a Siemens Elmiskop 101

* Corresponding author.
† Present address: Laboratoire de Genetique Moleculaire des Eucaryotes, Faculte de Medicine, 67085 Strasbourg Cedex, France.
‡ Present address: Biotechnology Australia Pty. Ltd., Roseville, New South Wales 2069, Australia.

TABLE 1. Bacterial strains, plasmids, and phages

Sample	Relevant characteristics	Reference
Bacterial strains		
Escherichia coli K-12 C600	Leu$^-$ Thi$^-$ Thr$^-$	2
Escherichia coli K-12 ED24	Spc^r	67
Escherichia coli K-12 ED395		19
Escherichia coli K-12 ED3818	His$^-$ Lys$^-$ Trp$^-$ Str^r Nal^r	68
Escherichia coli K-12 ED3886	RecA$^-$ Spc^r	*recA56* derivative of ED24
Escherichia coli K-12 ED8654	Met$^-$	6
Escherichia coli K-12 JC3272	His$^-$ Lys$^-$ Trp$^-$ Str^r	1
Escherichia coli K-12 JC6310	His$^-$ Lys$^-$ Trp$^-$ Str^r RecA$^-$	64
Escherichia coli B		59
Enterobacter aerogenes (ATCC 13048)		54
Proteus morganii (ATCC 25830)		54
Providencia stuartii 164		55
Salmonella typhimurium LT2 (NCIB 10248)		13
Shigella sonnei		J. Govan (personal communication)
Pseudomonas aeruginosa PAO2	Ser$^-$	28
Plasmids		
pBR322Δ::IS*1*	pBR322 deletion plasmid carrying IS*1*	M. Chandler (personal communication)
pCU1	IncN Ap^r Spc^r	35
pED899	Sul^s Tra$^-$ R46	This paper
pED904	in vitro deletion of R46 containing *Hin*dIII A fragment	8
pED1012	pBR322 derivatives containing N3 *Bgl*II E fragment in the *Bam*HI site	This paper
pED1013	As for pED1012, but with N3 *Bgl*II E fragment in opposite orientation	This paper
pED1017	Tra$^-$ N3	This paper
pED1022	pME420::IS*46*(d)	This paper
pED1030	R46::pME420 cointegrate	This paper
F		27
pIP1091	pBR322 derivative carrying IS*15*Δ	36
pME420	pBR325 derivative with a 1.0-kb deletion of the Tc^r gene removing the *Hin*dIII, *Bam*HI, and *Sal*I sites	J. Watson (personal communication)
pML31	*Eco*RI f5 fragment of F linked to the *Eco*RI fragment of R6-5 carrying Km^r	60
N3	IncN Sul^r Spc^r Tc^r *Eco*RII Uvp$^+$	61
R1-19	IncFII Ap^r Cm^r Km^r Sm^r Sul^r	46
R46	IncN Ap^r Sul^r Spc^r Tc^r Uvp$^+$	16
R68	IncP Ap^r Km^r Tc^r	10
R100	IncFII Cm^r Hg^r Sm^r SuT^r Tc^r	21
R388	IncW Sul^r Tp^r	17
R390	IncN Ap^r Spc^r Sul^r Tc^r *Eco*RII	14
RSF1010	IncQ Sm^r Sul^r	24
Phages		
EDλ4	λ *b515 b519 c*I857 *Sam7*	19
VAλ3	EDλ4 *exo*::IS*1*	66
P1 *vir* a	Virulent variant of P1 *kc*	65

electron microscope. Molecules were measured with a Ferranti Cetec digitizer with an Olivetti P6040 minicomputer.

RESULTS

R46 contains short regions of homology. Linear single-stranded molecules of R46 were obtained by cleaving R46 DNA at its unique *Xho*I site and denaturing at high pH. After a brief period of reannealing, the molecules were spread and photographed in the electron microscope. The structures observed (e.g., Fig. 1) showed that R46 contains two pairs of inverted repeat sequences. One pair formed a short double-stranded stem of ca. 340 bp with a single-stranded loop of ca. 7.3 kb. The dimension and location of this stem-loop structure indicate that it is equivalent to that previously observed by Langer et al. (39) on pKM101, a deletion mutant of R46 (47). This pair of short inverted repeats flanks the *mucAB* genes (51) and possibly the replication region of the two plasmids (Fig. 2A).

The second pair of inverted repeats formed a double-stranded region of 0.86 ± 0.03 kb and were separated by ca. 9.4 kb of DNA which forms the two single-stranded "tails" in Fig. 1. One of these tails is very short, indicating that one copy of the repeat lies very close to the *Xho*I site in the region of R46 that is deleted in pKM101 (Fig. 2A; 8). This second pair of inverted repeats would not, therefore, have been detected by Langer et al. (39). The position of each copy of this repeat on R46 coincided with closely spaced cleavage sites for *Sal*I and *Pst*I, suggesting that these sites are contained within the repeated sequence.

Inversion of a region of R46 flanked by inverted repeats. The sizes of the restriction endonuclease cleavage fragments of R46 DNA have been measured independently by Brown and Willetts (8) and by Langer and Walker (40), and there were striking differences between some of the values obtained by the two groups. The existence of the second pair of inverted repeats described above could account for these discrepancies, since recombination between the two copies of this sequence would invert the intervening 9.5-kb segment, producing two distinct forms of R46. We designated our form "R46A" and designated that of Langer and Walker "R46B" and confirmed the differences in restriction fragment sizes by direct comparison on agarose gels (Fig. 3). R46A and R46B had similar total sizes and contained the same total number of cleavage sites for each restriction enzyme used. For *Kpn*I and *Sma*I, for which there are no

sites within the 9.5-kb segment, the digest patterns were identical, whereas for others (*Bam*HI, *Bgl*II, *Eco*RI, and *Hin*dIII) most fragments were common, but two fragments in each case differed between R46A and R46B. For example, the 17.1- and 8.5-kb *Bgl*II A and B fragments of R46A were replaced by fragments of 22.0 and 3.6 kb in R46B. Analysis of the sizes and the map positions of such altered fragments confirmed that the two forms of R46 differ by inversion of the 9.5-kb segment. This inversion most probably results from reciprocal recombination between the homologous inverted repeats. Although the inverted segment contains sites for *Sal*I and *Pst*I, the restriction digest patterns for these two enzymes were indistinguishable between R46A and R46B. This further supports the inference that the repeated sequence itself contains a site for each of these enzymes (Fig. 2A).

Evidence for similar short regions of homology on N3. A restriction endonuclease cleavage map of the IncN plasmid N3 was constructed by techniques similar to those used for R46 (8). A combined physical and genetic map of N3 is presented in Fig. 2B; a description of the data from which it was derived will be presented elsewhere (G. Coupland, A. Brown, and N. Willetts, manuscript in preparation).

The map shows that there are two pairs of *Sal*I and *Pst*I sites (characteristic of the inverted repeat sequences found in R46) in N3, though repeated in direct, rather than inverted, orientation. Both small *Sal*I-*Pst*I fragments from N3 were shown to comigrate on acrylamide gels with the *Sal*I-*Pst*I fragment originating from near coordinate 36 kb in R46. This suggested that N3 carries two copies of a repeated sequence similar to those found on R46. Further evidence for this resulted from consideration of heteroduplex molecules formed between single strands of N3 and R46; in these molecules a short (0.7 ± 0.05 kb) region of heteroduplex DNA formation was apparent which corresponds in position to a part of one of the R46 repeat sequences, and to a complementary region on N3 located between coordinates 51 to 52 kb, that includes both *Sal*I and *Pst*I sites (Fig. 2B; Coupland et al., in preparation).

During the course of our studies of N3, a spontaneous Tc^s deletion mutant (pED991) was detected fortuitously. *Bgl*II digests showed that ca. 11 kb of DNA had been lost, and *Pst*I digests showed that the endpoints of the deletion lie close to the *Pst*I sites at coordinates 51.75 and 5.4 kb (Fig. 2B). No novel-sized *Pst*I or *Sal*I fragments were present, suggesting that pED991 arose by homologous recombination between the directly repeated sequences covering these coordinates, leading to excision of the intervening DNA.

The four repeated sequences on R46 and N3 are shown below to have the transposition properties of an insertion sequence. We shall therefore refer to them as IS*46*(a) and IS*46*(b) (mapping near coordinates 36 and 46 kb on R46) and IS*46*(c) and IS*46*(d) (near coordinates 51 and 5 kb on N3), respectively.

IS*46* will fuse R46 or N3 with a second plasmid to form cointegrate molecules. One consequence of the presence of an insertion sequence in a plasmid is that it will fuse this plasmid with another replicon to form a cointegrate molecule with directly repeated copies of the insertion sequence present at each interreplicon boundary (25, 50). Such cointegrates are often resolved into their component plasmids at only low frequencies, even in Rec^+ hosts (4, 23). Consequently, conjugative plasmids carrying insertion sequences can mobilize nonconjugative plasmids as part of cointegrate molecules. We used this system to determine whether the four repeated sequences decribed in the previous section were transposable. An insertion sequence may also transpose directly, but this is very difficult to measure and was not attempted in the present case.

A *recA* host strain (JC6310) carrying R46 or N3, and the monomeric form of the nonconjugative plasmid pME420 (a Cm^r Tc^s deletion derivative of pBR325), was used as the donor in matings with the Rec^+ Nal^r recipient strain ED3818. The frequencies of formation of Tc^r [Nal^r] and Cm^r [Nal^r] transconjugants were taken as measures of R46 or N3 and pME420 transfer, respectively.

The strain containing R46 and pME420 gave Cm^r transconjugants at a frequency of 3×10^{-5} relative to R46

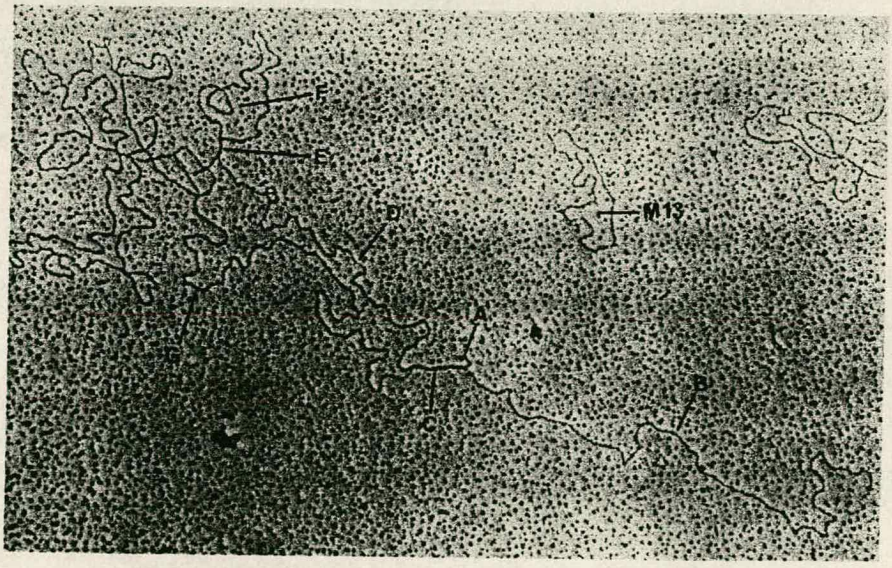

FIG. 1. Self-annealed R46 single-strand DNA after cleavage of the plasmid with *Xho*I. Two double-stranded "snap-back" regions are visible, of lengths 0.86 and 0.34 kb, labeled C and E, respectively. The lengths in kilobases of the single-stranded segments of the molecule are as follows: A, 0.06; B, 9.4; D, 7.6; F, 7.3; and G, 23.8. A single-stranded M13 size standard is marked.

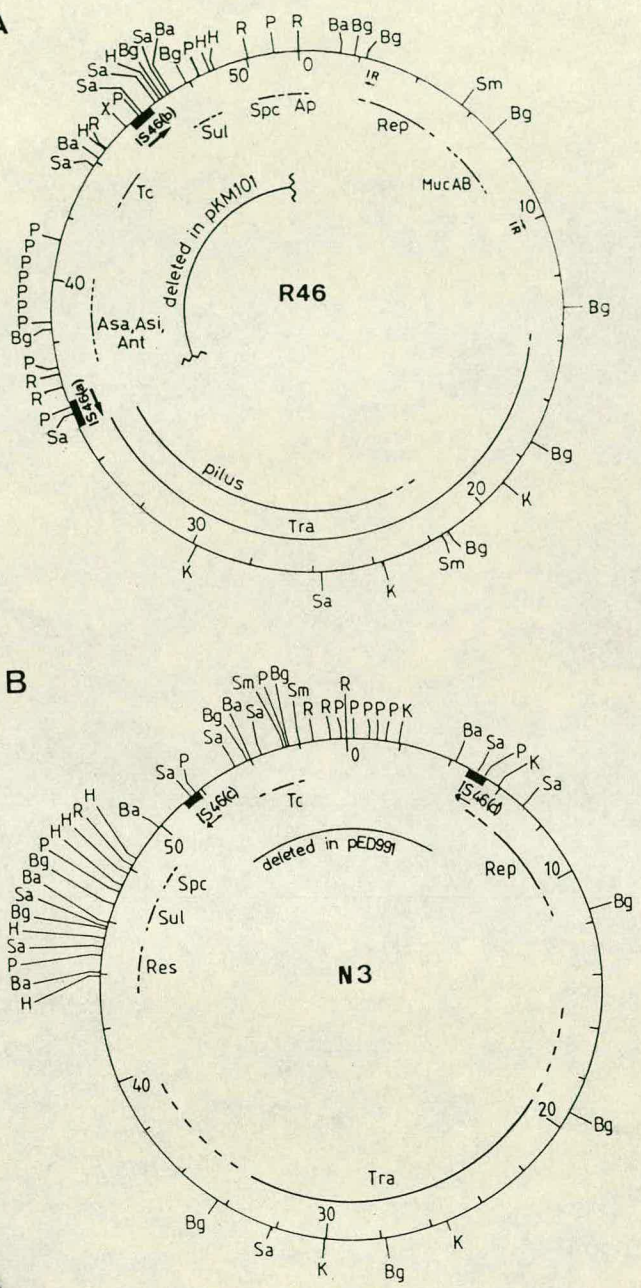

FIG. 2. (A) A map of R46. Redrawn from Brown and Willetts (8) with the addition of the two copies of IS*46* (drawn with heavy lines), and of the 0.34-kb inverted repeats (I.R.). Abbreviations are as follows: Ap, ampicillin; Asa, arsenate; Asi, arsenite; Ant, antimony; MucAB, enhanced mutagenesis; Rep, replication; Spc, spectinomycin; Sul, sulphonamide; Tc, tetracycline; and Tra, conjugal transfer. As well as the previously reported arsenate resistance, R46 confers resistance to arsenite ions and antimony III. The restriction enzyme cleavage sites shown are *Eco*RI (R), *Hin*dIII (H), *Bgl*II (Bg), *Pst*I(P), *Sal*I(S), *Bam*HI (Ba), *Kpn*I (K), *Sma*I (Sm), and *Xho*I (X). Coordinates are marked in kilobases. Map coordinates of restriction fragments referred to in the text are as follows: *Bgl*II-A, 21.3 to 38.4; *Bgl*II-B, 38.4 to 46.9; and *Pst*I-F, 36.0 to 37.2. (B) A map of N3. A detailed description of the data that allowed construction of this map will be presented elsewhere (Coupland et al., in preparation). The phenotype of N3 differs from that of R46 in that it does not carry Ap^r, Asa^r, Asi^r, or Ant^r determinants, its Tc^r gene is different (8, 45), and it encodes the *Hsp*II restriction and modification system. The transfer: all of these showed the antibiotic resistance phenotypes of both plasmids. Small-scale plasmid DNA preparations were made from 15 independent transconjugants and were digested with *Bgl*II. Cointegrates resulting from transposition of IS*46*(a) and IS*46*(b) were expected to have pME420 integrated within the *Bgl*II B and *Bgl*II A fragments of R46, respectively. In nine of the isolates, *Bgl*II-B had been replaced by a larger fragment (pME420 contains no site for *Bgl*II), whereas in the other six it was *Bgl*II-A that was replaced. This suggested that IS*46*(a) and IS*46*(b) could each promote cointegrate formation (i.e., transpose) at approximately similar frequencies. *Sal*I digests showed that the cointegrate plasmids contained the normal complement of R46 *Sal*I fragments, plus an extra 5.3-kb fragment arising from pME420 (which has no *Sal*I site) flanked by directly repeated copies of IS*46*. One of the IS*46* elements has therefore been duplicated in each case. These data confirmed that the Cm^r transconjugants contained R46::pME420 cointegrates formed by transposition of IS*46*. Restriction digests of two representative cointegrates formed via IS*46*(a) and IS*46*(b) are shown in Fig. 4A.

In analogous experiments, pED904 was substituted for R46. pED904 is an in vitro deletion mutant of R46 (8), which carries IS*46*(a) but not IS*46*(b). This plasmid mobilized pME420 at a frequency of 8×10^{-6} relative to its own transfer, and *Bgl*II digests of plasmid DNA from Cm^r transconjugants showed them to contain pED904::pME420 cointegrates formed via IS*46*(a). IS*46*(a) is therefore capable of cointegrate formation (i.e., transposition) in the absence of IS*46*(b). A derivative of R46 carrying IS*46*(b) alone was not available to allow the independent transposition of this copy to be tested.

N3 also mobilized pME420, at a frequency of 1.5×10^{-5} of its own transfer. Plasmid DNA from 12 independent Cm^r transconjugants was examined by digestion with *Bgl*II and *Sal*I, and all 12 carried cointegrates of N3 and pME420 formed by transposition of IS*46*(d) (Fig. 4A). This copy of IS*46*, therefore, transposed more frequently than IS*46*(c) in these experiments, although results in the following section show that IS*46*(c) is also capable of transposition.

Cointegrate formation between R388 and *tra*$^-$ derivatives of R46 or N3. Although the mobilization technique used above provides an effective test for the presence of insertion sequences on conjugative plasmids, it would not detect direct transposition of antibiotic resistance markers by a mechanism independent of cointegrate formation. Since the tetracycline resistance markers in both R46 and N3 are flanked by copies of IS*46* (Fig. 2A), and these combinations might form composite transposons, we wished to look for direct transposition of these. The conjugation protocol was therefore reversed by using R388 (a conjugative IncW Tp^r Sul^r plasmid with no known transposable sequences) to mobilize the Tc^r markers of transfer-deficient mutants of R46 and N3.

A JC6310 derivative carrying R388 and pED889 (a *tra*$^-$ point mutant of R46 with a residual transfer frequency of $<10^{-7}$) transferred the pED889 Tc^r marker to ED3818 at a frequency of 10^{-6} compared with R388 transfer. Each transconjugant carried all of the pED889 antibiotic resistance markers plus Tp^r, suggesting that they carried cointegrates

copies of IS*46* are marked with heavy lines. Abbreviations are as for Fig. 2A; Res, restriction modification. Map coordinates of fragments mentioned in the text are as follows: *Bgl*II-A, 55.4 to 11.7; and *Bgl*II-E, 46.6 to 53.9.

of the two plasmids. This was confirmed by *Bgl*II digestion of plasmid DNA from six representative transconjugants, and the fragment pattern further showed that one cointegrate has arisen via IS*46*(a) transposition, and five have arisen via IS*46*(b) transposition. Digests of two cointegrates are shown in Fig. 4B. A large number of the Tc^r transconjugants from the above mating were then screened to search for occasional transposition of the segment flanked by IS*46*(a) and IS*46*(b) as a Tc^r and As^r transposon. Of 350 Tc^r colonies tested, 3 were Tp^r Ap^s Spc^s, the pattern expected to result from direct transposition of such a transposon. However *Bgl*II restriction analysis showed that all three plasmids carried much larger segments of R46 DNA and were deletion derivatives of cointegrate molecules. These data, plus the observation that none of the 21 cointegrates examined contained duplications of the entire IS*46*(a)-IS*46*(b) region, indicate that transposition of this region occurs (if at all) considerably less frequently ($<10^{-9}$ per R388 transconjugant) than that of IS*46* itself.

Similar experiments were carried out for N3, using pED1017, a *tra*$^-$ N3 mutant deficient in pilus formation with a residual transfer frequency of 5×10^{-7}. A JC6310 derivative carrying R388 and pED1017 transferred the Tc^r marker of the latter plasmid at a frequency of 9×10^{-6}, compared with R388 transfer. All of the transconjugants carried all of the pED1017 antibiotic resistance markers as well as Tp^r, but in most, pED1017 and R388 were present as separate plasmids. This may to be due to a low level of complementation of the transfer defect of pED1017 by R388. Clones carrying cointegrate plasmids were identified amongst the transconjugants as those which could retransfer Tc^r at high

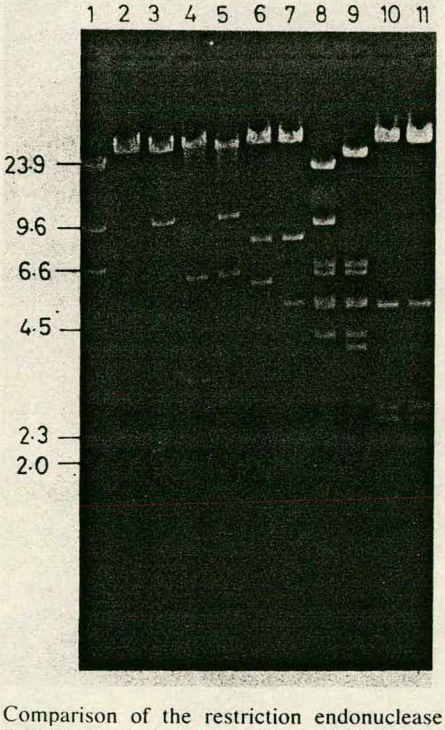

FIG. 3. Comparison of the restriction endonuclease cleavage patterns of R46A (lanes 2, 4, 6, 8, and 10) and R46B (lanes 3, 5, 7, 9, and 11) cleaved with *Hin*dIII (lanes 2 and 3), *Bam*HI (lanes 4 and 5), *Eco*RI (lanes 6 and 7), *Bgl*II (lanes 8 and 9), and *Pst*I (lanes 10 and 11). Size standard in lane 1 was produced by cleaving λ DNA with *Hin*dIII.

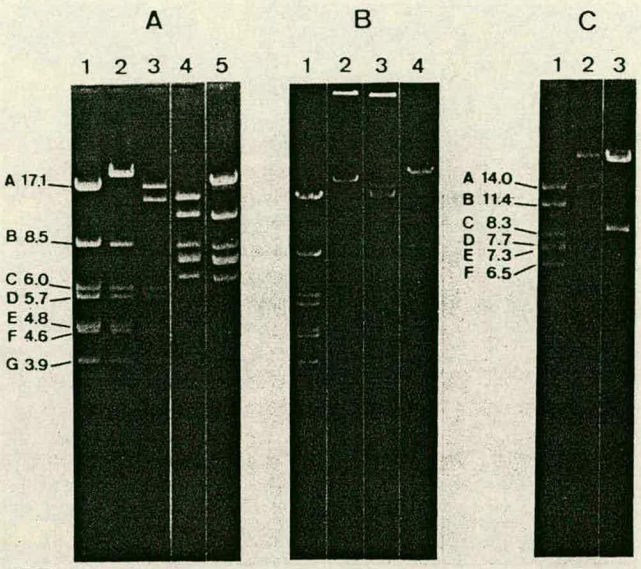

FIG. 4. *Bgl*II restriction analysis of cointegrate plasmids formed via various copies of IS*46*. (A) Lanes: 1, R46; 2, an R46::pME420 cointegrate formed via IS*46*(a); 3, an R46::pME420 cointegrate formed via IS*46*(b); 4, N3; and 5, an N3::pME420 cointegrate formed via IS*46*(d). (B) Lanes: 1, pED889; 2, a pED889::R388 cointegrate formed via IS*46*(a); 3, a pED889::R388 cointegrate formed via IS*46*(b); and 4, R388. (C) Lanes: 1, pED1017; 2, a pED1017::R388 cointegrate formed via IS*46*(c); and 3, R388. The DNA in all lanes was digested with *Bgl*II. pME420 contains no site for this enzyme, whereas R388 has two *Bgl*II sites, giving fragments of 24 and 8.6 kb. The sizes of the *Bgl*II fragments of R46 and of pED1017 (same pattern as N3) are marked in kilobases. The fragments carrying copies of IS*46* are as follows: R46 *Bgl*II-A contains IS*46*(a), R46 *Bgl*II-B contains IS*46*(b), N3 *Bgl*II-E contains IS*46*(c), and N3 *Bgl*II-A contains IS*46*(d).

frequency in replica matings with ED24. They constituted ca. 6% of the initial Spc^r Tc^r Tp^r transconjugants. DNA from 11 of these was digested with *Bgl*II to confirm that they were cointegrates: of these, 8 were formed via IS*46*(c), and three were formed via IS*46*(d) (Fig. 4B). In these matings there was no evidence for the independent transposition of Tc^r or of duplication in the cointegrates of the entire IS*46*(c)-Tc^r-IS*46*(d) region of N3.

In other experiments R388 mobilized the Ap^r genes of either pED1012 or pED1013 at frequencies of 5×10^{-6}. These plasmids are pBR322 derivatives containing the *Bgl*II E fragment of N3, which carries IS*46*(c), cloned in either orientation. *Bam*HI and *Sal*I digestion of plasmid DNA prepared from these transconjugants showed the patterns expected for R388::pED1012 or R388::pED1013 cointegrates resulting from IS*46*(c) transposition. IS*46*(c) transposition is therefore independent of the presence of IS*46*(d).

Resolution of R46::pME420 cointegrates. R46::pME420 cointegrates obtained as described in the previous section were used to determine whether IS*46*-mediated cointegrates could be resolved by either a plasmid- or host-encoded mechanism and consequently to provide pME420::IS*46* plasmids for studies of retransposition. Initial experiments with two cointegrates, formed via IS*46*(a) and IS*46*(b), were unsuccessful in that attempts to separate the expected Cm^r Tc^s pME420::IS*46* resolution product by either transformation or P1 transduction failed. However, it was possible that these cointegrates had been formed by R46 insertion within the replication region of pME420, so that the pME420::IS*46*

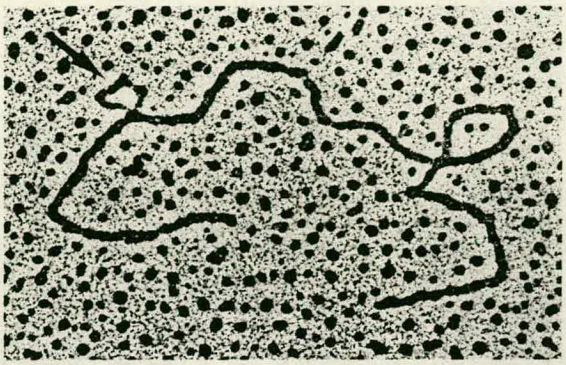

FIG. 5. A heteroduplex molecule formed between pML31 and pED994 after cleavage at their *Eco*RI sites. The 0.8-kb single-stranded loop (marked with an arrow) represents the insertion of IS*46*(d) present in pED994.

resolution product would be inviable. This had been observed previously for an IS*21*-based cointegrate (66). Because of the large size of the cointegrate molecule, it was difficult to demonstrate unequivocally that this was indeed the case, although *Pst*I digestions indicated that it was possibly so (data not shown). *Pst*I digestions of other cointegrates allowed one to be chosen (pED1030) in which integration had taken place [via IS*46*(a)] at a site ca. 2 kb from the *Pst*I site of pME420 and therefore definitely not in the replication region.

*Pst*I digestion of pED1030 DNA provided preliminary evidence that resolution was occurring in the Rec$^+$ host ED3818, since visual inspection of the gels indicated that those fragments derived from the expected (high copy number) pME420::IS*46*(a) component were present in greater molar amounts than those derived from the (low copy number) R46 component; this had not been observed for the other cointegrates. pED1030 was transformed into JC3272 and into the isogenic RecA$^-$ strain JC6310, and Cm^r Tc^r colonies were purified. Continued presence of the cointegrate plasmid was confirmed by showing that the antibiotic resistance markers of R46 and pME420 were transferred in filter matings at similar frequencies and that their coinheritance was 100%. Plasmid DNA was then isolated from each strain before and after growth through ca. 100 generations and was used to transform ED8654. Cm^r transformants were selected and tested for coinheritance of Tc^r, and the sizes of any Cm^r Tc^s plasmids were measured on agarose gels. There was no cointegrate resolution in the RecA$^-$ host during this growth period, whereas in the Rec$^+$ host the proportion of Cm^r transformants that were Tc^s increased by ca. 70%. This technique for detecting resolution has the advantage that the frequency of formation of Cm^r Tc^s transformants is increased relative to that of Cm^r Tc^r ones by the multicopy nature and small size of the pME420::IS*46*(a) resolution product; however, it has the concomitant disadvantage that the rate of resolution cannot be meaningfully quantitated. In similar experiments, R388::pED1022 (see below) was found to resolve slowly in Rec$^+$ but not RecA$^-$ cells.

Retransposition of IS46(a) from pED1022. One pME420::IS*46*(a) plasmid from the previous experiment was chosen, shown to have inherited *Sal*I and *Pst*I sites of IS*46*(a), and numbered pED1022. It was then transformed into JC6310 (R388)$^+$ to study the retransposition of IS*46*(a) via cointegrate formation. In conjugation experiments between this strain and the *recA*$^-$ recipient ED3886, the Cm^r marker of pED1022 was transferred at 10^{-6} of the R388 transfer frequency. Plasmid DNA was prepared from four independent Cm^r Tp^r transconjugants, and digestion with *Sal*I confirmed that in each case it gave the pattern expected for R388::pED1022 cointegrates with a copy of IS*46*(a) at each boundary.

IS*46*(a) was therefore retransposable from pED1022, demonstrating that no genes required for its transposition are located elsewhere on the R46 plasmid from which it was originally derived.

Physical characterization of IS*46*. Before pME420 became available to us, we had used pML31 as the receptor plasmid to study IS*46* transposition. pML31 is a nonconjugative, Km^r, mini-F replicon that has previously been used in Tn*3*-mediated transposition experiments (15), although the Km^r gene is present within Tn*903*, which is transposable at low frequency (48). In these experiments, an N3::pML31 cointegrate was obtained that had resulted from IS*46*(d) transposition, and P1 transduction was used to obtain the pML31::IS*46*(d) resolution product of this cointegrate. One Km^r transductant (out of 186 tested) was Spc^s Sul^s Tc^s and carried a plasmid numbered pED994. Restriction analysis showed that it was ca. 0.8 kb bigger than pML31 and carried the extra *Pst*I and *Sal*I sites characteristic of IS*46*.

Restriction analysis of pED994 showed that IS*46*(d) was located within a 4.51-kb *Eco*RI-*Hin*dIII fragment. The equivalent fragment in pML31 measured 3.7 kb, indicating that 0.81 kb of DNA had been added. This value was similar to the length of DNA duplicated in N3::pME420 cointegrates and was taken to be an accurate measure of the total size of IS*46*(d). The orientation of IS*46*(d) within the *Eco*RI-*Hin*dIII fragment was determined from double digests, using *Eco*RI together with *Sal*I or *Pst*I, and the distance from the *Eco*RI site to the *Sal*I site within IS*46*(d) was accurately measured

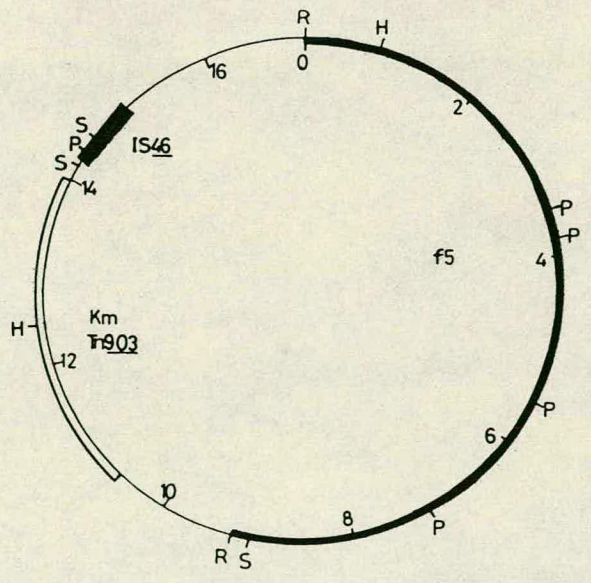

FIG. 6. A map of pED994, a pML31 plasmid carrying an insertion of IS*46*(d) (shown as a solid box). The restriction map of pML31 is based on the data of Kahn et al. (33) and Timmis et al. (60). The *Pst*I and *Sal*I sites of IS*46* are located 145 and 470 bp from the termini of the element, respectively, and the internal *Pst*I-*Sal*I distance is 195 bp. The restriction enzyme cleavage sites shown are *Eco*RI (R), *Pst*I (P), *Sal*I (S), and *Hin*dIII (H). The f5 *Eco*RI fragment derived from the plasmid F is shown as a thicker line, and the transposon Tn*903* is designated by double lines.

as 2.41 kb. The size of the internal *Sal*I-*Pst*I fragment of IS*46*(d) was 195 bp.

The distance from the same *Eco*RI site to the point of IS*46*(d) insertion was then determined by electron microscopy. Heteroduplex molecules formed between *Eco*RI-digested pED994 and pML31 DNA showed a single insertion loop corresponding to IS*46* located at 1.94 ± 0.04 kb (mean of 11 molecules) from the *Eco*RI site (Fig. 5). The distance from one terminus of IS*46*(d) to the internal *Sal*I site is, consequently, 2.41 − 1.94 = 0.47 kb, and from the internal *Pst*I site to the other terminus is 810 − 470 − 195 = 145 bp. The size of the single-stranded loop in the heteroduplexes was measured as 0.78 ± 0.02 kb (mean of 10 molecules), which is in good agreement with the size of IS*46*(d) determined from restriction enzyme digests. No double-stranded "stem" bounding this loop was visible, suggesting that if inverted repeat sequences are present at the termini of IS*46*, they are probably shorter than 100 bp. Maps of IS*46*(d) and pED994 are shown in Fig. 6.

The four copies of IS*46* show homology to each other and to IS*15* but not to IS*1*. To demonstrate that the four copies of IS*46* in R46 and N3 are homologous, the two plasmids were digested with *Bgl*II, and the fragments were separated on agarose gels and transferred to nitrocellulose filters (57). Duplicate filters were then hybridized to nick-translated, ^{32}P-labeled pED994 or pML31 (control) probe DNA. pED994 hybridized equally strongly to each of the four *Bgl*II fragments carrying separately IS*46*(a), IS*46*(b), IS*46*(c), and IS*46*(d), thus confirming the close similarity of all four repeated sequences (Fig. 7A).

Further hybridization experiments were carried out to demonstrate the nonhomology of IS*46* with IS*1*, an insertion sequence of similar size. First, pED994 did not hybridize to the *Eco*RI fragment of VAλ3 (EDλ4*exo*::IS*1*) that includes IS*1* (Fig. 7B). Second, ^{32}P-labeled pBR322Δ::IS*1* DNA did not hybridize to three of the four *Bgl*II fragments of R46 and N3 which carry copies of IS*46*, although it did to the fourth (data not shown). In a control experiment ^{32}P-labeled pBR322 DNA also hybridized strongly to the fourth fragment, *Bgl*II-B of R46; this fragment contains the Tc^r determinant of R46, known to be closely related to that of pBR322 (8). Strong hybridization of pBR322Δ::IS*1* to the appropriate *Eco*RI fragment of VAλ3 confirmed that IS*1* hybridization could be detected in this experiment. We therefore conclude tht IS*46* and IS*1* are not closely related.

Recently, a new insertion sequence, IS*15*Δ, has been described that is similar in size to IS*1* but does not share DNA homology with it (36, 37). Since, like IS*46*, this has single *Pst*I and *Sal*I sites, we attempted to hybridize ^{32}P-labeled pIP1091, a pBR322 derivative carrying IS*15*Δ, to *Bgl*II-digested R46 and N3. Strong hybridization to the *Bgl*II-digested bands containing IS*46* confirmed that IS*46* and IS*15*Δ are indeed related (Fig. 7A). This finding is considered further below.

Search for copies of IS*46* on other plasmids and in bacterial chromosomes. Representative plasmids from six different incompatibility groups were screened for the presence of IS*46* or related sequences by Southern blot hybridization. Restriction digests of the plasmids F (IncFI), R1-19 (IncFII), R100 (IncFII), R68 (IncP), RSF1010 (IncQ), R388 (IncW), R390 (IncN), pCU1 (IncN), and R46 (IncN) were separated by electrophoresis in agarose gels and transferred to nitrocellulose filters. These were then hybridized with the internal *Sal*I-*Pst*I fragment of IS*46*(d) ^{32}P-labeled by nick translation. With the exception of the R46 control and pCU1, none of these plasmids exhibited homology with the IS*46* probe fragment (data not shown). pCU1 contains one pair of the closely spaced *Pst*I and *Sal*I sites characteristic of IS*46* (35).

Similarly, nitrocellulose blots of *Eco*RI-digested chromosomal DNA from a variety of species of the families *Enterobacteriaceae* and *Pseudomonadaceae* were hybridized with the IS*46*-specific probe. R46 DNA was used as a hybridization control on the same filters. The species tested were *E. coli*, *Salmonella typhimurium*, *Shigella sonnei*, *Proteus morganii*, *Serratia marcescens*, *Providencia stuartii*, *Enterobacter aerogenes*, and *Pseudomonas aeruginosa*. None of these chromosomal DNA preparations showed any significant homology with IS*46* (data not shown).

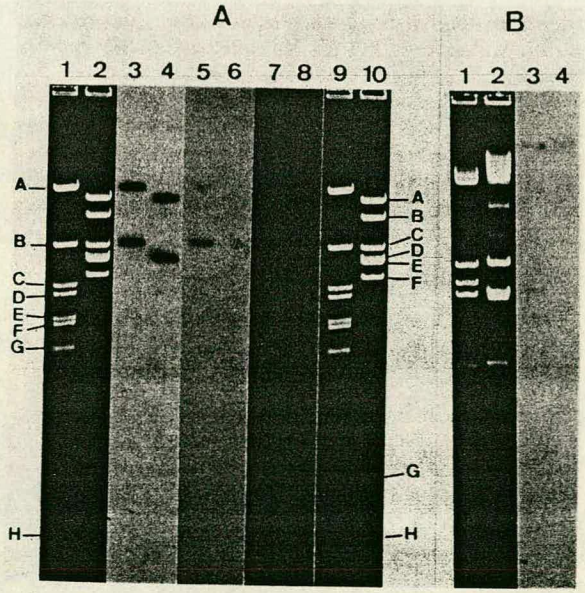

FIG. 7. (A) Gel photographs of the *Bgl*II cleavage fragments of R46 (lanes 1 and 9) and N3 (lanes 2 and 10) and autoradiographs of the same two lanes after Southern transfer and hybridization with ^{32}P-labeled pED994 (lanes 3 and 4), pML31 (lanes 5 and 6), or pIP1091 (lanes 7 and 8). The positions of the *Bgl*II restriction fragments of R46 and N3 are marked next to lanes 1 and 10, respectively. The weak hybridization of pML31 to *Bgl*II-B of R46 (lane 5) was unexpected and is not understood, whereas the stronger hybridization of pIP1091 to this fragment (lane 7) than to others containing IS*46* is due to the identical Tc^r determinants of R46 and pIP1091. (B) Gel photographs of the *Eco*RI fragments of EDλ4 (lane 1) and VAλ3 (lane 2) and autoradiographs after Southern transfer and hybridization with ^{32}P-labeled pED994.

DISCUSSION

We have shown that the conjugative IncN plasmids R46 and N3 each contain two copies of a sequence, designated IS*46*, which has the transposition properties of an insertion sequence. All four copies of IS*46* displayed homology in hybridization experiments and contained a common *Sal*I-*Pst*I internal fragment of ca. 195 bp; we therefore conclude that they are closely related, if not identical.

The transposition of insertion sequences results in either insertion of a discrete copy of the element within a target replicon or fusion of the donor and target replicons to form a cointegrate molecule with a copy of the element at each boundary (for a review, see ref. 34). A model whereby these two different transposition products result from diverging

alternative transposition pathways has been proposed (22, 26). Our assay of IS*46* transposition was based upon cointegrate formation and consequent conjugative mobilization either of the Tc^s pBR325 derivative pME420 by the IncN plasmid or of a *tra*$^-$ IncN plasmid mutant by the transposon-free IncW plasmid R388. Cointegrate formation took place in a RecA$^-$ host strain, as expected for an insertion sequence-based mechanism. Restriction enzyme cleavage analysis of the cointegrates showed that cointegration had occurred at a variety of sites on the recipient replicon and that a copy of IS*46* was present at each interreplicon boundary. Both findings are again characteristic of cointegrate formation via insertion sequence transposition. In addition, such analysis showed which copy of IS*46* was involved. Taking the results overall, each of the four copies of IS*46* transposed with approximately similar frequencies; these varied from 10^{-7} to 10^{-5}, according to which mobilization assay system was used. None of the other inverted repeat sequences on R46 (Fig. 1 and 2; 39) or N3 (data not shown; Coupland et al., in preparation) were found to be duplicated in any of these cointegrates, so that either these are transposable at frequencies that are one to two orders of magnitude less than that of IS*46* or are not transposable at all.

Resolution of both R46::pME420 and R388::pED1022 cointegrates was followed in Rec$^+$ and RecA$^-$ strains; it took place only in the Rec$^+$ host. In common with most other insertion sequences and in contrast to γ-δ and Tn*3* then (34), IS*46* does not encode a site-specific recombination system giving efficient resolution of cointegrate molecules. Such resolution therefore relies upon reciprocal recombination between the two IS*46* copies, brought about by the host *recA*-dependent, generalized recombination system, which is relatively inefficient for such short regions of homology (4, 23). One of the pME420::IS*46*(a) resolution products formed in a Rec$^+$ host was used to demonstrate that IS*46*(a) could be retransposed to R388, forming a cointegrate, and hence that IS*46*(a) transposition is independent of other R46 DNA sequences.

Dodd and Bennett (20) have recently described a resolution site (*res*) located on the *Pst*I F fragment of R46, which contains one terminus of IS*46*(a). However, the absence of resolution of pME420::R46 cointegrates in RecA$^-$ cells suggests that this *res* site is unlikely to be a part of IS*46*. Furthermore, pED1022::R388 cointegrates were stable in RecA$^-$ cells even in the presence of R46, showing that the R46 resolution system which will act in trans to resolve R46::Tn*3* cointegrates will not resolve cointegrates formed via IS*46*.

A further property manifested by insertion sequences is the deletion of adjacent segments of DNA. Such deletions extend outwards from the last nucleotide of the transposable element for variable distances, such that the element itself is not deleted (58). The plasmid pKM101 is an As^s Tc^s Sul^s Spc^s deletion derivative of R46 (Fig. 2) that is included in bacterial strains used for the Ames test for the detection of carcinogens as mutagens (44). Although derived by two consecutive deletion events (47), pKM101 differs from R46 by the absence of a single contiguous DNA segment of 15 kb (8, 40). One of the endpoints of this deleted region lies at, or extremely close to, IS*46*(a), and it is most likely that the deletions that formed pKM101 were generated by this element. Similarly, a fortuitously isolated transfer-deficient R46 mutant was shown to have a deletion extending into the transfer region from a point at or near IS*46*(a).

The two copies of IS*46* present on both R46 and N3 provide short regions of DNA homology across which intramolecular recombination may take place. On R46 the IS*46* elements are in opposite orientations, and recombination leads to inversion of the intervening segment, accounting for the existence of the two distinct forms of the R46 molecule designated R46A and R46B. In contrast, on N3 the IS*46* copies are directly repeated so that recombination would result in excision of the region between them. Its restriction fragment pattern showed that pED991, a spontaneous Tc^s deletion derivative of N3, is probably the product of such an excision. The loss of Tc^r from pED991 would then be analogous to the excision of resistance-determinant regions bounded by directly repeated copies of IS*1* from IncFII R factors in *S. typhimurium* and *Proteus mirabilis* (29, 62).

A number of well-characterized transposons contain insertion elements at their termini (34). The transposition functions of such "compound" transposons are encoded entirely by one or both terminal elements, which are, in most cases, independently transposable themselves. Moreover, it seems likely that any segment of DNA flanked by active insertion sequences in either orientation is potentially transposable. Examples for the short insertion sequence IS*1* include Tn*9* (42), Tn*1681* (56), Tn*2350* (11), and Tn*2571* (31). The region of R46 flanked by copies of IS*46* contains the determinants for Tc^r and As^r, whereas in N3 the intervening segment contains a Tc^r determinant. None of the transposition-mediated R46::pME420 or N3::pME420 cointegrates analyzed showed duplications of these larger regions, so we investigated the possibility that their transposition might preferentially result in simple insertions, rather than replicon fusions. No such Tc^r insertions into R338 were detected, however, and if generated at all, they must have been present at $<3 \times 10^{-9}$ per cell. Similarly, we were unable to detect transposition to R388 of the Tc^r region of N3. These results may simply reflect the long distance between the two IS*46* elements in each case: Chandler et al. (9) showed that for IS*1*-flanked composite transposons, there was a twofold decrease in the frequency of transposition for each extra kilobase of DNA between the insertion sequences. In the present case, this would give an approximately 1,000-fold reduction, so that transposition of Tc^r would be essentially undetectable.

Physical characterization of IS*46* showed that it is ca. 810 bp in length and contains single restriction sites for *Sal*I and *Pst*I that are located 470 and 145 bp from the termini, respectively. It contained no sites for *Eco*RI, *Hin*dIII, *Bam*HI, *Bgl*II, *Kpn*I, *Sma*I, or *Xho*I.

The restriction enzyme cleavage pattern plus the inability of IS*46* to hybridize to plasmids containing IS*1* (pBR322Δ::IS*1*, R100, and R1-19), IS*2* (F), IS*3* (F), γ-δ (F), IS*10* (R100), IS*21* (R68), or Tn*1* (1-19) showed that it is distinct from these transposable elements as well as from IS*4*, IS*5*, IS*50*, and IS*903* (34).

However, IS*46* seems to be related to the insertion sequences IS*15* (1.5 kb) and IS*15*Δ, IS*26*, and IS*140* (all 0.8 kb) in that all of these contain single *Sal*I and *Pst*I sites, and cross-hybridization between various members of the group has been demonstrated (7, 32, 36–38). Three composite transposons consisting of a Km^r gene flanked by direct repeats of IS*26* (Tn*2680*), IS*15* (Tn*1525*), and an IS*15*-like element (Tn*6*) have been described previously (3, 32, 36), whereas a single copy of IS*140* was adjacent to a Gm^r gene in two other plasmids (7). No association of IS*46*-like elements with Tc^r genes has been reported previously. IS*15*Δ (830 bp) results from the spontaneous loss of 670 bp of duplicated DNA from IS*15* (1,500 bp), and this loss is

associated with a reduction in the frequency of transposition from 5×10^{-5} to a frequency similar to that found for IS*46* (36). This reduction in frequency, together with the reduction due to the length of DNA between the flanking insertion elements (9; see above), is probably responsible for inability to detect transposition of Tn*2680* (J. Altenbuchner [7]) as well as the potential Tc^r transposons of R46 or N3. Transposition of Tn*1525*, flanked by IS*15* itself was measurable in a similar system.

Taken together, the above publications show that IS*46*-related elements are present on plasmids belonging to a variety of incompatibility groups, including IncA (RA1), IncC (pIP1031 and R40a), IncF (pJR62 and R124; we showed that it was not present on F, R100, or R1-19), IncI (several, including pIP112, pIP565, and R144), IncM (pIP135, pIP151, pTH1), IncT (Rts1), and IncY (pIP231), as well as on the IncN plasmids R46, N3, and pCU1. Unexpectedly, we found that IS*46* did not occur on the IncN plasmid R390, despite its similarity to other IncN plasmids and the presence of a Tc^r gene. Despite this wide distribution amongst bacterial plasmids, we were unable to demonstrate the presence of IS*46* on the chromosomes of several species of enterobacteria or *P. aeruginosa*: this contrasts to the other small insertion sequence, IS*1*, which although not found in *Pseudomonas* strains (66), is fairly widely distributed amongst the *Enterobacteriaceae* family (49, 53).

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