

Molecular and Genetic Studies of
the IncN Plasmids R46 and N3

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A Thesis presented for the Degree of
Doctor of Philosophy

Department of Molecular Biology

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October 1981



FOR DIANA

ABSTRACT

The conjugative plasmids R46 and N3 are members of the incompatibility group N in E.coli, and confer resistance to ampicillin (R46 only), spectinomycin, sulphonamides, tetracycline and arsenate ions (R46 only). In addition, R46 was shown to determine resistance to arsenite and antimony (III) compounds. Both plasmids confer protection against UV-irradiation, and N3 determines the EcoRII restriction-modification system. Restriction endonuclease cleavage maps of R46 and N3 were derived, and a number of deletion and recombinant derivatives of both plasmids constructed. The phenotypes of these derivatives, taken in conjunction with their restriction fragment composition, allowed the construction of combined physical and genetic maps of R46 and N3. The regions necessary for plasmid replication and conjugal transfer were located, as well as the genetic determinants of the antibiotic and metal ion resistances, UV-protection, and the EcoRII phenotype.

Although poorly transmissible in broth, R46 and N3 were transferred with high efficiency on solid media. All the R46 DNA required for transfer was located within a single region of less than 22.5 kb, and recombinant clones containing this segment were self-transmissible. A cis-acting DNA sequence presumed to be the origin of transfer was located at or close to one end of the R46 transfer region.

A number of inverted repeat sequences were detected on both R46 and N3, and evidence was obtained that each plasmid contains two copies of a novel transposable element, designated ISN. In R46 these are in inverted orientation, while direct repeats of the element

may be present in N3. The isolate of R46 investigated by Langer and Walker (1981) differs from that studied here by inversion of the DNA segment between the two copies of ISN. The R46 derivative pKM101, used in the Ames test for the detection of carcinogens as mutagens, was probably produced by transposition-mediated deletions from the terminus of an ISN element.

Declaration

I hereby declare that I alone have composed this thesis, and that, except where stated, the work presented within is my own.

ACKNOWLEDGEMENTS

I am indebted to several members of this Department, past and present, for advice, suggestions, technical help, and profitable discussions. In particular I wish to thank Doug Johnson, John Watson, Roger Everett, Wendy Smith, John Maule, Andy Newman, Pierre Meulien, Keith Derbyshire, Barbara Will, Karam Singh, Philip Lehrbach and George Coupland.

I am especially grateful to Neil Willetts for excellent supervision, instruction and useful criticism; also to David Finnegan for helpful advice on many occasions, the generous provision of reliable enzymes, and for valuable comments on the manuscript of this thesis.

In addition I would like to thank Pam Beattie for her many hours spent at the electron microscope, Anna Nowosielska, Betty McCready and Jo Rennie for their co-operation with typing and photographic work, and Helen Bradley for proof-reading.

Finally I wish to acknowledge Nora Hunter, Anne Bowcock and Pierre Meulien for their friendship and moral support during the course of this work.

Abbreviations Used

Ap	ampicillin
Ant	antimony (III)
Asa	arsenate
Asi	arsenite
bp	base pair
Cm	chloramphenicol
hr	hour
kb	kilobase
Km	kanamycin
min	minute
Nal	naldixic acid
Rep	replication
Res	restriction (<u>EcoRII</u>)
s	second
Sfx	surface exclusion
Sm	streptomycin
Spc	spectinomycin
Sul	sulphonamide
Tc	tetracycline
Tp	trimethoprim
Tra	transfer
Uvp	ultraviolet protection

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CHAPTER 1 INTRODUCTION

(a) General Introduction(i) The Diversity of Plasmids

In recent years the widespread occurrence of plasmids in bacterial populations has become increasingly apparent. The first plasmid to be identified as an extrachromosomal genetic element was the sex factor F in E.coli; this was recognised by its ability to promote exchange of genetic information between bacterial strains by conjugation (Lederberg, 1952; Hayes, 1953). After the advent of antibiotics in the treatment of bacterial infections, pathogenic strains of Shigella dysenteriae were isolated in Japan which had acquired resistance to two or more antibiotics, apparently in a single step. This multiple drug resistance was shown to be determined by plasmids or 'R factors' some of which conferred resistance simultaneously to chloramphenicol, streptomycin, sulphonamides, and tetracycline, and which could be transmitted to other species of the Enterobacteriaceae (see Watanabe, 1963). Following the extensive medical and veter^{er}inary use of antibiotics, large numbers of antibiotic-resistance plasmids have since been isolated from drug-resistant bacteria in many countries (see Novick, 1974; Appendix B of Bukhari et al, 1977).

Plasmids are found in numerous different bacterial genera, both gram positive and gram negative, and may determine a variety of other phenotypes besides antibiotic resistance. Examples of other plasmid-borne properties include resistance to toxic metal ions (Summers et al, 1978), production of bacteriocins (Reeves, 1972), degradation of xenobiotic compounds such as toluene (Williams and Murray, 1974) and naphthalene (Dunn and Gunsalus, 1973), production

of enterotoxins (So et al, 1975) and the induction of tumours in plants (Chilton et al, 1977). The majority of known plasmids were originally recognised by the phenotypes they conferred on their host cells. Modern techniques of cellular DNA purification, however, have also revealed 'cryptic' plasmids with no known phenotype, such as the Pl5A plasmid of E.coli (Cozzarelli et al, 1968) and several plasmids in Rhodopseudomonas capsulata (Scahill, 1981). Plasmid-like elements may also be present in eukaryotes, for example the 2 μ m plasmid of yeast (see Hartley and Donelson, 1980).

(ii) The Exploitation of Plasmids

Conjugative plasmids (see below) have long been exploited in the study of bacterial genetics for their ability to promote chromosomal recombination between strains, for example in Hfr crosses (see Hayes, 1968). Since the advent of in vitro recombination and molecular cloning techniques however, these and other plasmids have been increasingly used as tools for research in molecular biology, and in potential commercial applications of gene cloning (see Old and Primrose, 1980; Glover, 1980; Timmis, 1981, for reviews of in vitro genetic manipulation techniques). Plasmids can be highly suitable as DNA cloning vehicles by virtue of their small size, their selectable phenotypes (e.g. antibiotic resistance), and their ease of introduction into bacterial cells. One of the many uses of in vitro recombination techniques employing plasmids is in the study of plasmids themselves; experiments described in this thesis are examples of this.

Other applications of plasmid cloning techniques to molecular biology are too extensive to summarise here. Among the more

spectacular recent examples, however, are the expression in E.coli of DNA sequences coding for human hormones and interferon (Goeddel et al, 1978, 1979; Nagata et al, 1980), and the use of the Ti plasmid of Agrobacterium to introduce foreign genes into plant cells (see Schell et al, 1981).

Broad host range plasmid cloning vehicles have been used to introduce genes into novel hosts (e.g. Cannon and Postgate, 1976; Windass et al, 1980) and offer commercial possibilities for the expression of alien or manipulated genes in a wide range of bacterial species. Moreover hybrid cloning vehicles have recently been constructed (e.g. Beggs, 1978; Mulligan and Berg, 1980; O'Hare et al, 1981), partly of plasmid DNA and partly of eukaryotic or viral DNA. As well as replicating in E.coli, such molecules can be introduced into yeast or mammalian cells. In some cases the expression in eukaryotic cells of a bacterial gene on the cloning vehicle allows selection of the cells containing recombinant plasmids. Plasmid cloning vehicles of this sort offer new possibilities for genetic manipulation in eukaryotes.

(iii) Plasmid Replication and Incompatibility

Plasmids, by definition, can replicate autonomously in host cells; this process is controlled, so that the number of plasmid copies per host chromosome remains constant under given growth conditions. Electron microscope observations of replicative intermediates have established that plasmid DNA replication is initiated from fixed origins. These sites may be unique, as in ColE1 (Lovett et al, 1974) or multiple, as in R6K (Crosa et al, 1976), and replication may be either unidirectional or bidirectional, as in

ColEI and F respectively (Lovett et al, 1974; Eichenlaub et al, 1977). Whilst replication of plasmid DNA depends largely on host functions, plasmid-coded products are also involved. Typically these are required for the control of replication initiation and plasmid copy number. In addition to the replication origin(s) there may also be cis-acting DNA sequences necessary for replication control. (For recent reviews of plasmid replication systems see Kolter, 1981; Thomas, 1981; Timmis et al, 1981; Veltkamp and Stuitje, 1981; Lane, 1981.) Some plasmids also contain a cis-acting sequence, par, which is not essential for replication per se, but ensures accurate segregation of plasmid molecules into daughter cells at cell division (e.g. Meacock and Cohen, 1981). A par function is particularly important for the stable inheritance of low copy number plasmids, as these might frequently be lost if segregation occurred at random.

Certain pairs of plasmids cannot be maintained together stably in the same cell under non-selective conditions. This phenomenon, called incompatibility, is believed to occur between plasmids the copy number or segregation of which are controlled by common mechanisms. Mutual incompatibility is widely used as a criterion for the classification of plasmids, and may reflect genuine relationships between plasmid replication systems. There are now at least 28 incompatibility (Inc) groups for plasmids that can be maintained in E.coli (Jacob et al, 1977).

(iv) Relationships between Plasmids

Molecular hybridisation studies have established a general correlation between incompatibility grouping and the degree of

overall DNA homology between plasmids. Plasmids within the same Inc group usually share extensive (>70%) DNA homology (Guerry and Falkow, 1971; Ingram, 1973; Falkow et al, 1974), and this is borne out by electron microscope observations of heteroduplexed plasmid DNA (Sharp et al, 1973; Gorai et al, 1979). Conversely plasmids in different incompatibility groups generally display little or no significant homology. The primary exceptions to these general rules are plasmids in different sub-groups of the F and I incompatibility complexes, which although compatible, share extensive DNA homology (Sharp et al, 1973; Falkow et al, 1974). There are also reports of incompatible plasmids showing negligible homology (e.g. see Grindley et al, 1973; Bayley et al, 1979).

Comparisons of certain closely related plasmids have shown them to differ mainly by the presence or absence of discrete transposable DNA segments, sometimes containing antibiotic resistance genes (Kopecko et al, 1976; Brevet et al, 1977; Gorai et al, 1979). Conversely, widely different plasmids may contain regions of DNA homology due to the presence of almost identical transposable elements, such as members of the widely distributed group of ampicillin-resistance transposons related to Tn3 (Hedges and Jacob, 1974; Kopecko and Cohen, 1975; Heffron et al, 1975, 1977). Thus transposable elements probably play a major role in plasmid evolution. DNA rearrangements and translocations mediated by such elements allow the modular assembly of new combinations of antibiotic-resistance genes and other phenotypic determinants. The properties of transposable elements are considered in more detail in Chapter 7.

(v) Conjugation Systems

Plasmids provide bacteria with potential genetic variability which may be advantageous to their host cells in a changing environment. If plasmid genes were constantly required, however, selective pressure would favour their permanent chromosomal integration rather than their existence in independent replicons. Despite plasmid-coded mechanisms for stable inheritance, the organisation of plasmid genes as autonomous episomes may therefore allow the possibility of their loss from cells when no longer required. Plasmid-free cells would have a small growth rate advantage over those carrying superfluous plasmid DNA, and there is evidence for such plasmid loss in natural populations. For example in the absence of antibiotic therapy, R factors tend to disappear from bacterial populations in man (e.g. Price and Sleight, 1970). The relationship between plasmids and their hosts may thus be considered as a form of transient symbiosis.

Many plasmids, however, have the capacity to transfer themselves between bacterial strains, and in some cases between many different genera (Datta and Hedges, 1972). This 'horizontal' transmission of plasmids may be essential for their survival, given the potentially transient requirement for their presence in any one bacterial population.

Self-transmissible plasmids that bring about bacterial conjugation are termed 'conjugative'. All naturally occurring conjugative plasmids are relatively large (>35 kb), and many of their genes are involved in the transfer process. Many non-conjugative plasmids can be transmitted only in the presence of specific conjugative plasmids present in the same cell and are said to be 'mobilisable'

(see Clark and Warren, 1979). ColE1 is a typical example of a small mobilisable non-conjugative plasmid; it is efficiently mobilised by F, R64-11, and certain other conjugative plasmids (Clowes, 1963; Warren et al, 1979). The conjugal transmission of certain other small plasmids has not been demonstrated, e.g. NTP11 (Grindley and Kelly, 1976; K. Derbyshire, personal communication), but in these cases the appropriate conjugative plasmid required for their mobilisation may be undiscovered. It is possible that all naturally occurring plasmids are potentially transmissible by conjugation, at least in those groups of bacteria in which conjugation has been demonstrated.

The clinical significance of plasmid transfer is exemplified by the conjugal transmission of antibiotic-resistance plasmids of incompatibility group P, as certain members of this group are transmissible to all species of gram-negative bacteria so far tested (see Thomas, 1981), including many human pathogens. In conjunction with transposable genetic elements, such 'promiscuous' plasmids may be largely responsible for the widespread dissemination of antibiotic resistance among different genera. For example, very similar ampicillin resistance transposons have been identified in natural isolates of Pseudomonas, Escherichia, Salmonella, Proteus, Haemophilus and Neisseria (Heffron et al, 1977; Falkow et al, 1977). Antibiotic resistance genes harboured in harmless bacterial strains may thus have the potential of transferring to pathogenic species and impeding successful drug therapy.

The most extensively studied plasmid conjugation system is that of F-like plasmids (reviewed by Willetts and Skurray, 1980). Certain features of this transfer system are broadly similar to those of

plasmids of other incompatibility groups in E.coli, although in general these other systems are phenotypically distinct (see Willetts, 1977) and share little or no DNA homology (see above). At least 12 of the 22 or more F plasmid genes involved in conjugation are required for the biosynthesis of extracellular filamentous organelles called pili. These are necessary for the formation of mating pairs or aggregates of donor and recipient cells, and serve as adsorption sites for F-donor-specific bacteriophages (see Achtman and Skurray, 1977). Bradley (1980) has identified pili determined by conjugative plasmids of all known incompatibility groups in E.coli, and classified them into three morphological groups: thin flexible, thick flexible and rigid filaments. These pilus types are determined by plasmids within particular sets of incompatibility groups. The pili of plasmids within an incompatibility group are also related serologically, while those determined by members of different Inc groups are often unrelated by this criterion (Bradley, op.cit.).

The other major feature of the F transfer system is a mechanism for conjugal DNA metabolism. This involves transfer of a single pre-existing strand of plasmid DNA from the donor, the process being initiated at a specific DNA sequence, oriT, by a plasmid-determined endonuclease (Rupp and Ihler, 1968; Ohki and Tomizawa, 1968; Everett and Willetts, 1980). Similar mechanisms may pertain to other transfer systems (Vapnek et al, 1971), but in general there is no evidence for this.

Cells carrying F plasmids are poor recipients for further F transfer (Lederberg et al, 1952) since the formation of stable mating pairs with other F^+ cells is inhibited (see Achtman and Skurray, 1977). The plasmid genes responsible for this surface exclusion

function are distinct from those involved in pilus synthesis (Achtman et al, 1980). Conjugative plasmids of incompatibility groups I, P and N also exhibit surface exclusion towards plasmids of the same respective group (Meynell, 1969; Hedges and Jacob, 1974; Datta and Hedges, 1971), although Hedges and Datta (1972) showed this not to be the case for IncW plasmids.

Although the transfer functions of F are expressed constitutively, most naturally occurring F-like R factors determine a fertility inhibition system that represses their own transfer and that of an F plasmid present in the same cell (Finnegan and Willetts, 1972). Studies of pilus production, levels of pilus-specific phage sensitivity, and plasmid transfer frequencies indicate that both repressed and constitutive transfer systems are found among plasmids of other incompatibility groups (see Willetts, 1977; Bradley, 1980; Bradley et al, 1980).

(b) IncN Plasmids

(i) Historical Introduction

The earliest classification of R factors in E.coli divided them into two groups, fi^+ and fi^- (Watanabe et al, 1964a), depending on their ability to inhibit the fertility of the plasmid F (see above). All fi^+ R factors tested by Lawn et al (1967) and Meynell et al (1968) determined sensitivity to F-specific phage, and exhibited mutual surface exclusion and incompatibility. In contrast fi^- R factors were found to be heterogeneous. Certain pairs of fi^- R factors could co-exist stably (Khatoon and Iyer, 1971), and while many fi^- plasmids determined sensitivity to ColI-specific phage, others did not (Lawn et al, 1967; Datta and

Hedges, 1971). Three of the latter were R46, N3 and R15. On the basis of mutual surface exclusion and incompatibility Datta and Hedges (1971) ascribed these three plasmids to a separate group, subsequently defined by incompatibility alone and called IncN. Numerous other members of this group have since been identified (for examples see Jacob et al, 1977).

(ii) Host Range

IncN plasmids have been isolated in nature from species of Escherichia, Klebsiella, Proteus, Providencia, Salmonella, Shigella and Yersinia (see Jacob et al, 1977; Kimura et al, 1976). Many of these genera include serious human or animal pathogens. Tardif and Grant (1980) tested the host range of specific IncN plasmids and found them transmissible to many genera of the Enterobacteriaceae. Jacoby (1975, 1977) has reported that an IncN plasmid (R46) is also transmissible to Pseudomonas.

(iii) N pili, and Similarities between IncN and other Inc Groups

Sensitivity to the filamentous bacteriophage Ike (Khatoon and Iyer, 1972) is specific to cells containing IncN plasmids. By analogy with other plasmid-specific phages, the cellular receptors for Ike were expected to be plasmid-determined conjugation pili. Attempts to detect such pili were unsuccessful (Brodt et al, 1974), until Bradley (1979) obtained electron microscope photographs of those determined by N3, and demonstrated that they could adsorb IncN-specific phages. These 'N pili' were fragile, easily detached from cells in suspension and apparently synthesised constitutively (Bradley, 1979, 1980).

Several observations suggest possible relationships between

the transfer systems of IncN plasmids and those of incompatibility groups P, W, M and X. Plasmids of these four groups determine morphologically similar rigid pili (Bradley, 1980), and these show certain similarities in their bacteriophage adsorption specificities. Phage PR4 (Bradley and Rutherford, 1975) for example, adsorbs specifically to N, P and W pili (Bradley, 1976, 1979; / Bradley and Cohen, 1977), although sensitivity to PR4 has not been reported for cells containing IncM or IncX plasmids. The phages PRR1 and Pf3, however, appear to be specific for cells carrying IncP plasmids only (Bradley and Rutherford, 1975), at least in E.coli. Also the IncN-specific phage Ike can be propagated to a small extent by cells containing IncP plasmids, and a host range mutant of the phage can form plaques on such cells (Grant et al, 1978). Despite these common features, however, Bradley (1980) detected no serological cross-reactions between N, P, W, M and X pili.

The fertility inhibition relationships exhibited between IncN, IncP, IncW and IncX plasmids (Olsen and Shipley, 1975; see Chapter 3) may also be evidence of a possible relationship between their transfer systems. Studies of DNA homology between plasmids in these groups, however, have yielded conflicting results. Ingram (1973) reported over 50% homology between the IncN plasmid R390 and the IncP plasmid RP1, whereas Falkow et al (1974) found less than 10% homology between N3 and either RP4 (IncP) or Sa (IncW). The evidence for a significant evolutionary relationship between plasmids of these incompatibility groups is thus inconclusive.

(iv) Unusual Properties of IncN Plasmids

In addition to multiple antibiotic resistance, a common property

of IncN plasmids is the ability to confer protection against the lethal effects of UV-irradiation, and to enhance the rate of UV-induced mutagenesis in the host cell. These phenotypes, referred to as Uvp, are discussed in greater detail in Chapter 3. Similar properties are determined by members of several incompatibility groups in E.coli, but are most prevalent among IncN and IncI α plasmids (Molina et al, 1979). UV-protective and mutagenic plasmids are also found in Pseudomonas (Krishnapillai, 1975; Lehrbach et al, 1977).

Several IncN plasmids determine the EcoRII restriction-modification system (see Chapter 3), originally known as hspII (Bannister and Glover, 1968); this phenotype appears to be unique to members of the N incompatibility group (Hedges, 1972a).

Another distinctive property of certain IncN plasmids is their elimination from thy⁻ E.coli hosts under conditions of thymine starvation (Pinney and Smith, 1971). R factors of other incompatibility groups do not show this effect, and elimination is apparently confined to IncN plasmids lacking a functional EcoRII restriction determinant (Birks and Pinney, 1975; Tribe and Pinney, 1980). The causes of plasmid elimination and its relationship with EcoRII restriction are far from clear.

IncN plasmid conjugation and the phenotypes of R46 and N3 are considered in greater detail in Chapter 3.

The primary objectives of the work described in this thesis are the molecular and genetic characterisation of two representative IncN plasmids, R46 and N3, by construction of their physical and genetic maps. These data should enhance our understanding of the

organisation of genetic material in this important group of Enterobacterial plasmids, and will greatly assist further studies of the properties or features of these molecules. Two aspects of R46 are investigated in more detail: a cis-acting sequence (oriT) required for plasmid transfer, and the nature of transposable elements present in the plasmid.

CHAPTER 2 MATERIALS AND METHODS

(a) Growth Media and Buffers

L Broth contained, per litre: Difco Bacto Tryptone, 10 g; Difco Bacto yeast extract, 5 g; NaCl, 5g. pH 7.2.

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

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

Difco Nutrient Agar: Difco Bacto Nutrient Broth, 8g; Difco agar 15 g per litre.

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

LC Agar: Difco Bacto Tryptone, 10 g; Difco Bacto yeast extract, 5 g; NaCl, 5 g; Difco agar 10g; 0.5 M CaCl_2 , 4 ml; 20% (w/v) glucose, 5 ml; 0.25% (w/v) Thymidine, 4 ml, per litre, pH 7.2.

M9 Minimal Agar: Davis New Zealand agar, 20 g; NH_4Cl , 1 g; NaCl, 0.5 g; Na_2HPO_4 , 7 g; KH_2PO_4 , 3 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4 g; Glucose, 2.5 g; Thiamine, 2 μg per litre.

Difco top agar: Difco Bacto agar, 7 g; L Broth, 40 ml per litre.

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

LC top agar: Difco Bacto Tryptone, 10 g; Difco Bacto yeast extract, 5g; NaCl, 5 g; Difco agar, 7 g; 0.5 M CaCl_2 , 10 ml.

Buffers:

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

Bacterial buffer contained, per litre: KH_2PO_4 , 3 g; Na_2HPO_4 , 7 g; NaCl, 4 g; $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g.

TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA.

Table 2.1 Final concentration of additives to minimal and nutrient
media

<u>Antibiotics</u>	<u>Minimal</u> (µg/ml)	<u>Nutrient</u> (µg/ml)
Ampicillin (Penbritin)	50	40
Chloramphenicol (Chloromycetin)	50	50
Kanamycin (Kantrex)	25	20
Nalidixic Acid (P.L. Biochemicals)	40	40
Spectinomycin (Upjohn)	100	20
Streptomycin (Glaxo) (low level)	10	10
Streptomycin (Glaxo) (high level)	400	400
Sulphonamide (Sulphadimidine)	100	-
Tetracycline (Achromycin)	10	20
Trimethoprim (Sigma)	50	-
<u>Amino acids</u>	20	
<u>Others</u>		
Arsenate ($\text{Na}_2\text{HAsO}_4 \cdot 7\text{H}_2\text{O}$)	-	4 mg/ml (13 mM)
Arsenite (NaAsO_2)	-	250 µg/ml (2 mM)
Antimony ($\text{KSbO}_4 \cdot \text{H}_2\text{O}$)	-	8.1 µg/ml (250 µM)
Thymine	50	50

TES buffer: TE containing 1% (w/v) NaCl.

TAE buffer: 40 mM Tris, 20 mM sodium acetate (pH 8.2), 5 mM EDTA.

TBE buffer: 90 mM Tris, 89 mM boric acid, 2.5 mM EDTA.

(b) Plasmids, Bacteriophages, Escherichia coli K12 strains (see Tables 2.2, 2.3, 2.4).

(c) Conjugation techniques

(i) Quantitative Broth matings

Fresh overnight cultures of donor and recipient strains were diluted 1:20 in L broth, shaken at 37°C to a density of 2×10^8 cells/ml and chilled on ice. 0.2 ml of donor cell culture were added to 1.8 ml of recipient in a glass test-tube (18 mm x 150 mm) and left to stand in a water-bath at 37°C for 30 min. The culture was then chilled briefly on ice and diluted in phage buffer. 0.1 ml volumes of appropriate dilutions were added to 2 ml aliquots of molten Difco top agar (42°C) and poured over the surface of selective plates. These were of minimal agar with appropriate additives to select transconjugant cells. Donors were usually contraselected with nalidixic acid. Viable counts of donor cells were obtained by plating 0.1 ml of a 10^{-5} dilution of donor culture on nutrient agar. Colonies were counted after incubation for approximately 1 day (nutrient agar) and 2 days (minimal agar). Prior to counting, 25 donor cell colonies were patched on nutrient agar, grown overnight at 37°C, and replicated onto selective media to confirm the presence of the plasmid marker. When transfer of two different plasmids from the same donor strain was selected using different phenotypic markers, 50 transconjugants of each type were patched and replicated to determine the proportion that had received both plasmids.

(b) Table 2.2 Plasmids

<u>Plasmid</u>	<u>Phenotype, Genotype or Description</u>	<u>Incompatibility Group</u>	<u>Reference/Source</u>
R46 (R46A) ¹	Ap ^R Asa ^R Asi ^R Sm ^R /Spc ^R Sul ^R Tc ^R Uvp ⁺ Tra ⁺ Slo ⁺	IncN	Datta & Hedges, 1971 (N. Willetts)
R46 (R46B) ¹	Ap ^R Asa ^R Asi ^R Sm ^R /Spc ^R Sul ^R Tc ^R Uvp ⁺ Tra ⁺ Slo ⁺	IncN	Datta & Hedges, 1971 (G. Walker)
pED889	Tra ⁻ mutant of R46 (= pWS40)	IncN	W. Smith
pKM101	Ap ^R Uvp ⁺ Tra ⁺ Slo ⁺	IncN	Mortelmans & Stocker, 1979
N3	Sm ^R /Spc ^R Sul ^R Tc ^R Res ⁺ Mod ⁺ (<u>EcoRII</u>) Uvp ⁺ Tra ⁺	IncN	Watanabe <u>et al</u> , 1964a
R390	Ap ^R Cm ^R Sm ^R /Spc ^R Sul ^R Tc ^R Res ⁺ Mod ⁺ (<u>EcoRII</u>)	IncN	Coetzee <u>et al</u> , 1972
RP1	Ap ^R Km ^R Tc ^R Tra ⁺	IncP	Sykes & Richmond, 1970
pED748	Ap ^R Km ^R Tp ^R ; (Tc ^S RP1::Tn7 = pW70)	IncP	J. Watson
Sa	Cm ^R Sm ^R /Spc ^R Km ^R Sul ^R Tra ⁺	IncW	Watanabe <u>et al</u> , 1968
R388	Sul ^R Tp ^R Tra ⁺	IncW	Datta & Hedges, 1972
JCFLO	<u>Flac</u>	IncFI	Achtman <u>et al</u> , 1971
JCFL83	<u>Flac</u> <u>traD83</u>	IncFI	Achtman <u>et al</u> , 1971
EDFL171	<u>Flac</u> <u>traΔ337</u> , <u>traI⁻</u>	IncFI	McIntire & Willetts, 1978
JCFL102	<u>Flac</u> <u>traM102</u>	IncFI	Achtman <u>et al</u> , 1972
JCFL42	<u>Flac</u> <u>traG42</u>	IncFI	Achtman <u>et al</u> , 1971
JCFL79	<u>Flac</u> <u>traG79</u>	IncFI	Achtman <u>et al</u> , 1971
KPF48	<u>Flac</u> <u>traN548</u>	IncFI	Miki <u>et al</u> , 1978
JCFL1	<u>Flac</u> <u>traA1</u>	IncFI	Achtman <u>et al</u> , 1971

JCFL81	Flac traG81
pBR322	Ap ^R Tc ^R
pBR327	Ap ^R Tc ^R ; <u>bom</u> ⁻ Δ of pBR322
pAT153	Ap ^R Tc ^R ; <u>bom</u> ⁻ Δ of pBR322
pED825	Ap ^R ; Δ derivative of pBR322
pBR325	Ap ^R Cm ^R Tc ^R
pED815	Cm ^R Tc ^R ; (Ap ^S Δ derivative of pBR325)
pBR328	Ap ^R Cm ^R Tc ^R ; <u>bom</u> ⁻ Δ of pBR325
pSC101	Tc ^R
pML2	Km ^R ColE1 derivative
pML31	Km ^R mini-F replicon

IncFI	Achtman <u>et al</u> , 1971
	Bolivar <u>et al</u> , 1977
	Soberon <u>et al</u> , 1980
	Twigg & Sherratt, 1980
	R. Everett & N. Willetts (unpublished)
	Bolivar, 1978;
	Prentki <u>et al</u> , 1981
	Johnson & Willetts, 1980
	Soberon <u>et al</u> , 1980
	Cohen & Chang, 1977
	Hershfield <u>et al</u> , 1974
IncFI	Timmis <u>et al</u> , 1978

¹ See Chapter 4 for difference between R46A and R46B.

Table 2.3 Bacteriophages

<u>Bacteriophage</u>	<u>Genotype</u>	<u>Reference or Source</u>
Ike		Khatoon <u>et al</u> , 1972
PR4		Bradley and Rutherford, 1975
EDλ7	λ <u>c</u> I857 <u>Sam</u> 7	Willetts and McIntire, 1978
EDλ4	λ <u>b</u> 2 <u>c</u> ⁻	Dempsey and Willetts, 1976
λ467	λ <u>b</u> 221 <u>rex</u> ::Tn5 <u>c</u> I857 <u>O</u> 29 <u>p</u> 80	N. Kleckner
Pl vir a		Willetts <u>et al</u> , 1969

Table 2.4 Escherichia coli K12 strains

<u>Strain</u>	<u>Relevant Genotype</u>	<u>Reference or Source</u>
ED395	<u>lac</u> Δ U124	Dempsey and Willetts, 1976
JC3272	<u>his</u> <u>lys</u> <u>trp</u> <u>str</u> <u>gal</u> <u>lac</u> Δ X74	Achtman <u>et al</u> , 1971
ED3818	<u>nal</u> derivative of JC3272	Willetts and Maule, 1979
JC6310	<u>lys</u> ⁺ <u>recA</u> 56 derivative of JC3272	Willetts, 1975
ED2196	<u>his</u> <u>trp</u> <u>spc</u> <u>nal</u> <u>lac</u> Δ X74	Gasson and Willetts, 1977
ED8654	<u>supE</u> <u>supF</u> <u>metB</u> <u>hsdR</u> ⁻ <u>hsdM</u> ⁺	Borck <u>et al</u> , 1976
ED8641	<u>met</u> <u>trp</u> <u>hsdR</u> ⁻ <u>hsdM</u> ⁺ <u>recA</u>	N. Murray
AB1157	<u>argE3</u> <u>his</u> <u>leu</u> <u>pro</u> <u>thr</u> <u>str</u>	Howard-Flanders and Theriot, 1966
JG112	<u>polA1</u> <u>thy</u>	J. Gross
J5	<u>pro</u>	Bachmann, 1972
C600	<u>supE</u> ⁻	N. Willetts
J53	<u>pro</u> <u>met</u>	Bachmann, 1972
J62::Tn7	<u>his</u> <u>trp</u> <u>pro</u> (Tp ^R) <u>str</u> λ ⁺	M. Masters

(ii) Quantitative Filter Matings

Donor and recipient cultures were grown to a density of 2×10^8 cells/ml as above and chilled on ice. 0.2 ml of donor culture and 1.8 ml of recipient were mixed in a chilled 5 ml Bijoux bottle and drawn up into a 2 ml syringe through a hypodermic needle. The cells were then carefully filtered through a 20 mm diameter membrane filter (Millipore Corporation, pore size 0.45 μ m), and the filter transferred to a prewarmed nutrient agar plate. After incubation at 37°C for 30 min the filter was introduced into a 20 ml chilled Universal bottle containing 2 ml of fresh L broth, and the cells resuspended by vigorous agitation on a Whirlimixer for 30 sec. Appropriate dilutions were plated out in top agar, and the number of viable donor cells per ml determined, as described above. Donor colonies and transconjugants (where appropriate) were also checked for plasmid markers as described above.

(iii) Semi-quantitative Plate Matings

Parental cultures were grown overnight in L broth (static). 0.1 ml volumes of appropriate dilutions of donor cells and undiluted recipient culture were spread directly on transconjugant selective plates (streptomycin contraselection) and incubated overnight at 37°C. This method gave less efficient transfer of IncN plasmids than the filter mating method, but was sufficient, for example, to distinguish between Tra^+ and transfer-defective phenotypes.

(iv) Spot Tests for Mobilisable Plasmids

$\text{Tra}^- \text{Spc}^R \text{Tc}^S$ R46 derivatives were screened for mobilisation by pED912 ($\text{Tra}^+ \text{Tc}^R \text{Spc}^S$; see Chapter 5) using a triparental mating method. Minimal agar plates containing histidine, lysine, tryptophan, Spc and Sm (400 μ g/ml) were first spread with 0.1 ml of ED3818

(approximately 10^9 cells/ml) and then with 0.1 ml of ED395 (pED912) from a fresh overnight culture. The plate was left to dry briefly. Finally 0.01 ml spots of ED8654 strains containing the plasmids under test were applied to the surface of the agar and allowed to dry. After incubation at 37°C for 2 days the numbers of $\text{Sp}^{\text{R}} [\text{NaI}^{\text{R}}]$ transconjugant colonies in each spot were compared with those in spots of positive and negative control strains (ED8654 (pED923) and ED8654 (pED941) respectively, see Chapters 5 and 8).

(d) Bacteriophage Techniques

(i) Ike and PR4 Preparation and Spot Tests

0.1 ml of ED395 (R46) were mixed with phage in 2 ml of molten LC top agar and poured over an LC nutrient agar 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 4 ml L broth. The top agar was vortexed with this broth in a Universal bottle and the mixture centrifuged for 5 min (5,000 g). The supernatant was then carefully decanted and contaminating bacterial cells eliminated, either by treatment with Diethyl ether (for Ike) or by filtration (for PR4; pore size $0.22 \mu\text{m}$, Millipore Corporation).

For spot tests, 0.1 ml of static overnight culture were added to 0.25 ml of molten LC top agar, and poured onto LC nutrient agar. 0.01 ml 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 at 37°C .

(ii) Lambda Lysates and Titres, for Measurement of Plasmid-Determined Restriction Activity

Lambda plate lysates were prepared by a similar method to that described in (d) (i) above except that top agar was BBL (containing 1 mM MgSO_4) and wet L agar plates were used instead of LC agar. The final supernatant was treated with chloroform. For measurements of N3-determined restriction activity, dilutions of ED λ 4 grown on ED395 were titred on ED395 and ED395(N3) by mixing 0.1 ml phage with 0.1 ml bacterial culture (grown on L broth containing 2% maltose) in 2 ml molten BBL top agar, and pouring on BBL agar plates. Plaques were scored after overnight incubation at 37°C. For semi-quantitative restriction tests, 0.01 ml spots of serial dilutions of phage were applied to top agar containing the strains under test. After incubation plaques were counted within the spots where possible.

(iii) Pl Transduction

0.1 ml of Pl vir a (10^7 pfu/ml) were mixed with 0.2 ml 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 0.1 ml transducing phage lysate (10^8 pfu/ml) with 0.1 ml fresh overnight 'recipient' culture and 0.1 ml of 30 mM MgSO_4 , 15 mM CaCl_2 , incubating at 37°C for 20 min, and spreading on selective minimal agar (containing 1% (w/v) Na_3 citrate). Transductant colonies were purified 3 times on nutrient agar to remove excess Pl.

(e) Plasmid Phenotype Tests

(i) Arsenate and Arsenite Resistance

Fresh overnight cultures in L broth of ED395 strains containing

plasmids under test were diluted $\times 10^{-4}$ in phage buffer. 0.01 ml spots of these dilutions were applied to Oxoid nutrient agar plates containing sodium arsenate (4 mg/ml), sodium arsenite (280 μ g/ml), or no additive. After overnight incubation at 37°C, cell growth was compared with that of similar ED395 and ED395(R46) spots on the same plates. Strains judged to be sensitive to the metal ions were checked for retention of other plasmid markers.

(ii) UV-irradiation Experiments

AB1157 was used as host strain for all tests of plasmid-mediated UV-protection and enhanced MMS-mutagenesis. Plasmid-containing strains and AB1157 control were grown to 2×10^8 cells/ml, chilled, and diluted 1:100 in bacterial buffer. 5 ml of diluted cell suspension were transferred to a glass petri dish and exposed to doses of 0, 25, 50, 75 and 100 Jm^{-2} of short-wave ultraviolet light delivered at $10 \text{ Jm}^{-2} \text{ s}^{-1}$. UV fluences were measured with a Laterjet UV meter. Appropriate dilutions were added to Difco top agar, poured over Oxoid nutrient agar plates, and incubated overnight at 37°C.

(iii) MMS-induced Mutagenesis

Difco top agar was supplemented with histidine, leucine, proline and threonine at 1 mg/ml each, and arginine at 8 μ g/ml. To 2 ml aliquots of this agar (molten) were added 0.1 ml aliquots of fresh overnight cultures of AB1157 or AB1157 (R46), and 0, 5, 10 or 20 μ l volumes of 1% (v/v, in water) MMS (Methyl-MethaneSulphonate; Eastman). They were then plated on M9 minimal agar. After incubation for 5 days at 37°C, arg⁺ revertant colonies were counted. (The argE3 allele of AB1157 is an ochre mutation.) Parallel experiments using 1 mg/ml arginine in the same top agar confirmed that the volumes of MMS added had no significant killing effect.

(f) Plasmid DNA Preparations

(i) Small-scale Cleared Lysate Preparations for Screening Purposes

1.4 ml of stationary phase cultures were pelleted in 1.5 ml 'snap-cap' polypropylene microfuge tubes using an Eppendorf microfuge (30 sec). The cells were resuspended in 50 μ l lysis buffer (35 mM Tris-HCl, pH 8.0; 70 mM EDTA; 18% (w/v) sucrose; 1 mg/ml lysozyme (Sigma)) and incubated at 37°C for 5 min. 50 μ l of Triton lysis solution were added (0.2% Triton X-100 (v/v); 50 mM Tris-HCl, pH 8.0; 60 mM EDTA), mixed, and the tubes left to stand at room temperature for 10 min. The lysates were cleared by centrifuging (Eppendorf microfuge, 5 min) and the viscous pellets carefully removed with Pasteur pipettes. 40 μ l of supernatants were then mixed with 10 μ l cleared lysate loading buffer (SDS 5% (w/v); Ficoll 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 subsequently estimated from the mobility of super-coiled closed circular forms on the gel, with reference to super-coiled molecules of known size.

(ii) Small-scale Plasmid DNA Preparations (Birnboim and Doly, 1979)

1.4 ml of shaken overnight culture in a microfuge tube was centrifuged (Eppendorf microfuge, 30 sec) the pellet resuspended by vortexing in 100 μ l lysis solution (Tris-HCl 25 mM, pH 8.0; EDTA 10 mM; glucose, 50 mM; lysozyme (freshly added) 2 mg/ml), and left on ice. After 30 min, 200 μ l alkaline SDS denaturation solution (SDS 1% (w/v); NaOH, 0.2 M) were added and mixed. After 5 min on ice, 150 μ l sodium acetate (3M, pH 5.0) were added and mixed by inversion. A white precipitate was visible at this stage

and the preparation was left on ice for a further 60 min. After centrifuging for 5 min (Eppendorf microfuge, room temperature), 400 μ l of supernatant were carefully removed to a clean microfuge tube, mixed with 1.0 ml of EtOH (100%), and left at -70°C for 20 min. Centrifugation (Quickfit microfuge, 2 min, 4°C) was followed by redissolving the pellet in 100 μ l dilute sodium acetate (0.1 M, pH 6), and the addition of 200 μ l EtOH. After a further 20 min at -70°C and 2 min centrifugation at 4°C , the final pellet was dried in a vacuum desiccator (30 min) and resuspended in 50 μ l TE buffer at 0°C . The plasmid DNA recovered by this method (approximately 2 μ g) was sufficiently pure for restriction endonuclease digestion and for use in transformation.

(iii) Large Scale Purification of Plasmid DNA (Clewell and Helinski, 1970; Humphreys et al, 1975)

1 ml 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 (16,000 g, 10 min, 4°C) and resuspended in 10 ml 25% (w/v) sucrose in 50 mM Tris-HCl, pH 8.0. Maintaining the cells on ice, 1.5 ml of lysozyme solution (20 mg/ml in 250 mM EDTA, pH 8.0) were added, followed by 8 ml of 250 mM EDTA (pH 8.0). After each addition the cell suspension was swirled intermittently on ice for 5 min. 12 ml of Triton lysis solution (0.2% (v/v) Triton X-100; 50 mM Tris-HCl; 62.5 mM EDTA; pH 8.0) were then added, thoroughly mixed by drawing up and expelling from a 25 ml pipette, and the suspension left on ice (30 min) for lysis to occur. The lysate was cleared by centrifugation (27,000 g, 45 min, 4°C) and decanted into a 50 ml measuring cylinder (on ice). 3% (w/v) NaCl and 10% (w/v) PEG 600 (polyethylene glycol) were added,

dissolved by inversion, and the solution left overnight at 4°C. After pelleting by mild centrifugation (5,000 g, 2 min) the PEG-DNA precipitate was re-dissolved in 7 ml TES buffer, and transferred to a 15 ml glass tube (Corex) containing 11 gm CsCl and 0.5 ml ethidium bromide (EtBr) solution (10 mg/ml in water). Once dissolved, the solution was left on ice (15-30 mins) before centrifugation (12,000 g, 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, a further 0.2 ml EtBr (10 mg/ml) added, and the density of the solution adjusted to 1.59-1.61 g/ml (by addition of TES buffer).

Ultracentrifugation (90,000 g, 40-60 hrs, 15°C) was carried out in Beckman heat-seal polyallomer tubes in a 50 Ti rotor. The lower of the two fluorescent bands (containing supercoiled plasmid DNA), viewed under long-wave UV, was extracted through the side of the tube into a 1 ml syringe using a hypodermic needle. The solution was then extracted 3 times with n-butanol (pre-equilibrated against saturated CsCl solution) to remove EtBr, and dialysed extensively against TE buffer at 4°C. In some cases plasmid DNA was further purified by digestion with ribonuclease (1 µg/ml, 65°C, 30 min) followed by extraction with phenol, extraction with chloroform, and precipitation with EtOH.

Finally, DNA concentration and protein contamination of the solution were estimated from measurements of OD₂₆₀ and OD₂₈₀ of a ten-fold dilution, using a Unicam spectrophotometer. Protein contamination was considered negligible if the 260/280 ratio was >1.7.

TABLE 2.5 Reaction conditions for restriction endonucleases

<u>Restriction</u> <u>Endonuclease</u>	<u>Tris-HCl</u>		<u>NaCl</u>	<u>MgCl₂</u>	<u>Gelatin</u>	<u>2-mercaptoethanol</u>	<u>KCl</u>	<u>EDTA</u>
	<u>mM</u>	<u>pH</u>	<u>mM</u>	<u>mM</u>	<u>μg/ml</u>	<u>mM</u>	<u>mM</u>	<u>mM</u>
<u>AluI</u>	6	7.5	50	6	100			
<u>BamHI</u>	100	7.5		10				
<u>BglII</u>	12	7.5		12		12		
<u>EcoRI</u>	100	7.5	50	10	100			
<u>HaeII</u>	50	7.5		5		6		
<u>HaeIII</u>	50	7.5		5		6		
<u>HindIII</u>	6	7.5	50	6	100			
<u>HpaI</u>	20	7.4		10		6	20	
<u>KpnI</u>	6	7.5	6	6	100			
<u>MspI</u>	20	7.4		10		6		
<u>PstI</u>	100	7.5		10				
<u>PvuII</u>	6	7.5	60	6	100	6		
<u>SacI</u>	14	7.5	90	6		6		
<u>SalI</u>	8	7.5	150	6	100			0.2
<u>Sau3A</u>	6	7.5	50	6	100			
<u>SmaI</u>	15	8.0		6			15	
<u>TaqI</u>	6	7.5	6	6	100			
<u>XhoI</u>	6	7.4	150	6		6		
<u>XmaI</u>	6	7.5	25	6	100	6		

(g) DNA techniques

(i) Ethanol Precipitation of DNA

0.1 vol sodium acetate (3 M, pH 5.0) and 2 vols EtOH (100%) were added to DNA solutions, usually in a microfuge tube. DNA was precipitated at -70°C for 20-30 mins followed by centrifugation (Quickfit microfuge, 5 min, 4°C). Pellets were washed with EtOH, left at -70°C for a further 15 min, centrifuged again, and dried in a vacuum desiccator (30 min) prior to resuspension in TE buffer.

(ii) Restriction Endonuclease Digestions

Appropriate quantities of DNA, restriction endonuclease, 10x restriction buffer, and distilled H_2O were mixed thoroughly in a microfuge tube and incubated at 37°C (65°C for TaqI) for 1 hr. Final volumes were usually 30 μl . Digestion was stopped by incubating at 65°C for 10 min (or by phenol extraction for TaqI). Reaction conditions for different enzymes are given in Table 2.5. Restriction endonucleases were obtained from Boehringer Mannheim GmbH, Bethesda Research Laboratories Inc., New England Biolabs Inc., S. Bruce, J. Maule, A. Newman, N. Hunter and P. Meulien. PstI was purified by the method of Green et al, 1978.

(iii) In vitro Recombination of DNA Fragments

DNA restriction digests were diluted to 10-40 $\mu\text{g}/\text{ml}$ (final volume 90 μl) with 10 mM Tris-HCl, 0.1 M NaCl, pH 7.5. 10 μl of 10x T4 ligase cocktail (660 mM Tris-HCl (pH 7.5), 10 mM EDTA, 100 mM MgCl_2 , 100 mM DTT, 1 mM ATP) were added, followed by T4 ligase/^{1 unit per μg DNA,} (obtained from K. Mileham). Ligations were carried out overnight at 10°C and stored, if necessary, at -8°C . TaqI-digested DNA was ligated at a concentration of 200 $\mu\text{g}/\text{ml}$, with 10 mM ATP in the 10x ligase cocktail, and at 15°C overnight (otherwise as above).

(iv) Transformation of E.coli with DNA

1 ml of fresh overnight culture was diluted to 20 ml in L broth, grown to early log phase by shaking at 37°C, chilled, and pelleted (5,000 g, 5 min). The cells were resuspended in 10 ml ice-cold MgCl₂ (100 mM) and immediately centrifuged again (5,000 g, 5 min). After resuspension in 10 ml CaCl₂ (75 mM), they were then left on ice (20 min) before pelleting again (5,000 g, 2 min), and were finally resuspended in 1 ml 75 mM CaCl₂, 15% glycerol (v/v). The competent cells were either used immediately for transformation or stored at -70°C until needed. DNA for transformation was diluted to 0.1 ml with TE buffer, and added to 0.2 ml of competent cells on ice. After 30 min the cells were transferred to 42°C for a 5 min heat-shock. 0.5 ml L broth were then added and the cells shaken at 37°C for 1-16 hr before plating on selective media for transformants.

(v) End-labelling of DNA Fragments with [³²P]

1 µCi of [³²P]-labelled nucleoside triphosphate (either dCTP or dTTP, Amersham Radiochemicals) in ethanolic solution was dispensed into a microfuge tube containing 5 µl sterile H₂O, and dried in a vacuum dessicator. 1 µg of DNA fragments in 30 µl restriction buffer was added, together with the 3 remaining (unlabelled) dNTPs (50 µM final conc.), and Klenow polymerase (Boehringer). The mixture was incubated for 30 min at room temperature.

(h) Gel Electrophoresis

(i) Horizontal Agarose Gels

Agarose slab gels (15 cm x 25 cm; 0.7%-1.5% agarose (w/v) in

200 ml TAE buffer) were placed between two perspex tanks (each containing 500 ml TAE buffer), and absorbent wicks ('Access-matting', saturated with TAE buffer) were positioned at each end to make electrical contact between the gel and the buffer tanks. 30 μ l DNA samples were mixed with 20 μ l 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 150 V for 15 min. After this period the wells were again filled with TAE, the gel covered with a thin polythene sheet ('Saran wrap'), and electrophoresis resumed at 45 V for approximately 16 hrs. After running, the gel was stained with ethidium bromide (Sigma, 5 μ g/ml) for 15 min, and de-stained in H₂O for 60 min. DNA was visualised by placing the gel on a long-wave UV transilluminator (Ultraviolet Products Inc., 365 nm peak transmission). Photography was with Ilford FP4 film and an exposure time of 10 s through a red filter (Hoya R (25A)).

(ii) Polyacrylamide Gels (Maniatis et al, 1975) and Autoradiography

5% or 8% (w/v) acrylamide gels in TBE buffer were prepared by mixing appropriate volumes of 37.5% (w/v) acrylamide and 2% (w/v) bis-acrylamide stock solutions with 10x TBE and H₂O. The acrylamide to bis-acrylamide ratio was 29:1 (w/w) and the gel volume 80 ml. In some cases the gel also contained 5% (v/v) glycerol. 3 ml of 1.6% (w/v) ammonium persulphate (freshly prepared in H₂O) and 50 μ l TEMED (N,N,N',N'-tetramethylethylenediamine, Serva) were added to initiate polymerisation. The mixture was then immediately poured between two vertical glass gel plates (either 20 x 20 cm or

20 x 30 cm) separated by 1.5 mm 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 min allowed for polymerisation. The well-former and water-agar seal were then removed and the gel connected to a perspex gel apparatus containing 1 litre TBE buffer in each buffer tank. 15 μ l DNA samples (previously ethanol precipitated and resuspended in TE buffer) were mixed with 2 μ l loading buffer (0.25% (w/v) bromophenol blue, 0.25% (w/v) xylene cyanol FF, 50% (v/v) glycerol, in TBE) and loaded with a Hamilton microsyringe. Gels containing 5% glycerol were run overnight at 100 V; otherwise electrophoresis was typically at 300 V for 3-5 hrs. Gels were stained for 5 min in ethidium bromide (5 μ g/ml), de-stained for 10 min in H₂O, and DNA bands were visualised under shortwave overhead UV illumination (Mineralight C5, 254 nm peak transmission). Photography was by exposure to Ilford FP4 film for 5 min through a red filter (Hoya R (25A)).

For autoradiography of [³²P]-labelled DNA, gels were dried down onto Whatman 3 MM paper using a Bio-rad gel drier (2 hr), and exposed overnight against Fuji RX film at room temperature.

(iii) Estimation of DNA Fragment Sizes

In order to obtain accurate estimates of DNA restriction fragment sizes, gel photographs were scanned with a microdensitometer (Joyce, Loebel & Co.) and migration distances of DNA bands measured from the peaks of densitometer tracings. The migration distances of standard fragments (of known molecular weight) were plotted against the logarithm of their sizes (using semi-log graph paper)

and the sizes of unknown fragments on the same gel determined from the graph. The size standards were HindIII restriction fragments of ED λ 7 (23.9, 9.6, 6.64, 4.45, 2.29, 1.95, 0.59, 0.13 kb, Daniels et al, 1980) and various restriction products of pBR322 (Sutcliffe, 1978).

(i) Electron Microscopy

Heteroduplexing, self-annealing, 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 Siemens 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. ϕ X174^{RF form} (5.38 kb) and M13 (6.23 kb) molecules were included on the grids to provide double and single strand length standards respectively.

(j) Construction of ED575. (= ED395::Tn5)

A single plaque of λ 467 on E.coli C600 (see Table 2.3) was used to make a plate lysate on the same strain (the O⁻P⁻ amber mutations in λ 467 are suppressed in C600). 1 ml of this lysate (2×10^7 pfu/ml) was mixed with 1 ml of ED395 culture (2×10^8 cells/ml), left at room temperature for 30 min and plated on nutrient agar containing kanamycin. After overnight incubation at 37°C, three colonies were obtained. The phage cannot replicate in ED395 and cannot form lysogens at 37°C, so Km^R colonies were expected to contain Tn5 inserted in the host chromosome. One such colony was purified, and spot tests with λ vir and λ cI confirmed that the strain (ED575) was not-lysogenic for λ .

(k) Construction of Cloning Vehicles

(i) pED931, a cloning vehicle for *Kpn*I fragments

The plasmid pML2 is a 13.2 kb Km^R ColE1 derivative containing 2 *Kpn*I restriction cleavage sites, one of which lies within a 3 kb *Xma*I restriction fragment. pML2 DNA was digested with *Xma*I, the fragments ligated, and used to transform ED8654 with selection for Km^R . This selection ensured the recovery of plasmids containing at least 3 *Xma*I fragments from pML2 (one carrying the replication region, and two together providing the Km^R determinant). One such plasmid, pED931, was approximately 10 kb in size. The 3 kb *Xma*I fragment of pML2 is not present in pED931, which therefore contains a single *Kpn*I cleavage site. This site is suitable for the insertion of *Kpn*I restriction fragments, and pED931 was used as a vehicle for cloning the *Kpn*I-C fragment of R46 (Table 5.3). pED931 contains the *hom* and *MobA* regions of ColE1 ((Warren *et al*, 1979) but is not significantly mobilised by R46 (data not shown).

(ii) pED961, a cloning vehicle for *Bgl*III fragments

pED923 DNA (Table 5.1) and pBR322 DNA were both digested with *Sal*I and *Eco*RI (sequentially), mixed, ligated and used to transform ED8654. Sul^R transformants were selected on M9 minimal agar containing methionine and *Sul*, and one such transformant contained the recombinant plasmid pED961. Restriction analysis of pED961 DNA showed it to be composed of a 2.8 kb *Eco*RI-*Sal*I restriction fragment of pED923, and the 3.6 kb *Eco*RI-*Sal*I fragment of pBR322 which includes the replication region and Ap^R determinant. The 2.8 kb fragment contains the Sul^R determinant of pED923 (from R46) and includes a single cleavage site for *Bgl*III. Insertion of *Bgl*III fragments at this site inactivates Sul^R (see Ch. 6.b.ii) so pED961 is suitable for use as an Ap^R Sul^R *Bgl*III cloning vehicle.

CHAPTER 3 PHENOTYPES AND PROPERTIES OF R46 AND N3

(a) Introduction

R46 was isolated from a strain of Salmonella typhimurium in Brighton in 1962. It was initially named R1818 (Datta and Kontomichalou, 1965; Lawn et al, 1967) and has also been referred to as R-Brighton (Drabble and Stocker, 1968). The plasmid TP120 (Grindley et al, 1972), isolated from the same strain of S.typhimurium as R1818, is probably identical to R46. R46 determines resistance to the antibiotics ampicillin, sulphonamide, tetracycline, streptomycin (Datta and Kontomichalou, 1965) and spectinomycin (Hedges, 1972b), and to arsenate ions (Summers et al, 1978). In addition it is self-transmissible (Datta and Kontomichalou, 1965), confers protection against the lethal effects of ultraviolet (UV)-irradiation (Drabble and Stocker, 1968), and causes slow growth of host cells on minimal media (Langer et al, 1981).

N3 was one of the first R factors identified as such, originating from clinical isolates of Shigella dysenteriae following a dysentery epidemic in Japan in the 1950s (see Watanabe, 1963). The plasmid confers resistance to sulphonamides, tetracycline, streptomycin et al (Watanabe/1964a) and spectinomycin (Hedges, 1972b), and is self-transmissible (Watanabe/et al 1964a). N3 also protects its host cell from the lethal effects of UV-irradiation (Babudri and Monti-Bragadin, 1977) and determines a specific restriction and modification activity (Watanabe et al, 1964a).

Previous investigations of the phenotypes of R46 and N3 are summarised below, in many cases supplemented with data from the present study. A separate section is devoted to experiments concerned with conjugal transfer.

(b) Phenotypes of R46 and N3

(i) Resistance to Ampicillin, Spectinomycin, and Streptomycin

The β -lactamase produced by the ampicillin resistance determinant of R46 differs markedly^k in substrate profile from the common TEM-type enzymes, particularly in its high activity against oxacillin (Datta and Kontomichalou, 1965). Mathew and Hedges (1976) distinguished 3 sub-classes of oxacillin-hydrolysing enzymes by iso-electric focussing, and showed R46 to produce an enzyme of the OXA-2 variety. They also found OXA-2 determinants on plasmids of incompatibility groups O, and P, as well as N, although most IncN plasmids tested produced TEM-1 β -lactamases. The OXA-2 enzyme of R46 is also unusual in being dimeric (Dale and Smith, 1976), and was the first β -lactamase reported to be composed of sub-units.

The spectinomycin and low-level streptomycin resistances of both R46 and N3 are determined by a single gene product, streptomycin/spectinomycin adenylate synthetase (Benveniste et al, 1970; Hedges, 1972b). Throughout this thesis spectinomycin resistance is therefore assumed to denote resistance to streptomycin also.

(ii) Resistance to arsenate, arsenite and antimony (III)

Introduction: (For clarity this section is divided into two parts.).

Summers et al (1978) reported that R46 could confer resistance to arsenate ions in both E.coli and Pseudomonas aeruginosa. Although other arsenate resistance plasmids, studied in E.coli and Staphylococcus aureus, are known additionally to determine resistance to arsenite and antimony (III) (see Silver et al, 1981), R46-containing strains have not previously been tested with these compounds.

Results: Spot tests of ED395 (R46) and ED395 on nutrient agar containing 2 mM sodium arsenite (see Ch. 2.e.i.) established that R46 does indeed confer resistance to arsenite, at a similar level to that observed for other plasmids (Silver et al, 1981). When testing for antimony resistance, Silver et al (op.cit.) found significant killing of plasmid-free E.coli K12 strains by less than 100 μ M antimony(III), but when ED395 (R46) and ED395 were applied to Oxoid nutrient agar containing up to 200 μ M antimony(III), no growth inhibition of either strain was observed. Moreover, three other plasmid-free strains, ED3818, AB1157 and J53, from different sub-lines of E.coli K12 were also apparently resistant to antimony(III) in these tests.

Difco nutrient agar (a medium used by Silver et al) was then prepared, containing various concentrations of antimony(III). On this medium the growth of ED395 was severely inhibited by 100 μ M antimony(III), and R46 conferred significant resistance to the compound. Difco nutrient agar containing 250 μ M antimony(III) was subsequently adopted as the best diagnostic medium for tests of Ant^R phenotypes. Silver et al (1981) observed a similar apparent resistance to antimony(III) for plasmid-free E.coli strains in certain media. It is not known whether components of these media, and of Oxoid nutrient agar, are responsible for the protective effect, or if components of Difco and other media used by Silver et al may perhaps assist antimony killing.

ED395 (N3) determined no detectable resistance to arsenate, arsenite, or antimony(III).

(iii) The Slo phenotype

The presence of R46 in certain strains causes a substantially

decreased growth rate on minimal media, an effect known as the Slo phenotype (Langer et al, 1981). The appearance of colonies on minimal agar is delayed by 12-24 hr and colonies of variable size are produced. Waleh and Stocker (1981) have recently demonstrated that this slow growth rate can be overcome by the addition of purines to the medium, but the reason for a plasmid-determined purine requirement is not known. No such effect on cell growth was observed for N3 (unpublished observations).

(iv) N3-mediated restriction and modification

Watanabe et al (1964a) first demonstrated that N3 reduced the efficiency of plating (e.o.p.) of phages λ and T1, and that the phages underwent modification in cells containing the plasmid. In agreement with these results, the e.o.p. of unmodified phage λ (E λ 4 grown on ED395) on ED395 (N3) was 6.5×10^{-3} relative to the e.o.p. on ED395. The N3 restriction-modification specificity, initially called hspII (Bannister and Glover, 1968; Hedges, 1972a), is now known as the EcoRII restriction-modification system (see Roulland-Dussoix et al, 1974). EcoRII restriction enzyme has been purified from strains containing the IncN plasmid R1551 and is commercially available. It is a type II restriction endonuclease which recognises the DNA sequence $^{\dagger}CC(\overset{A}{T})GG$ (Bigger et al, 1973).

(v) The Uvp phenotypes

R46 protects its host cell from the lethal effects of UV-irradiation (Drabble and Stocker, 1968; see Fig. 3.1a). This is one of several plasmid-borne phenotypes related to the repair of UV-damaged DNA, which are probably mediated by a single plasmid gene product, and are here referred to as the Uvp phenotypes.

These phenotypes are expressed in E.coli and Salmonella typhimurium

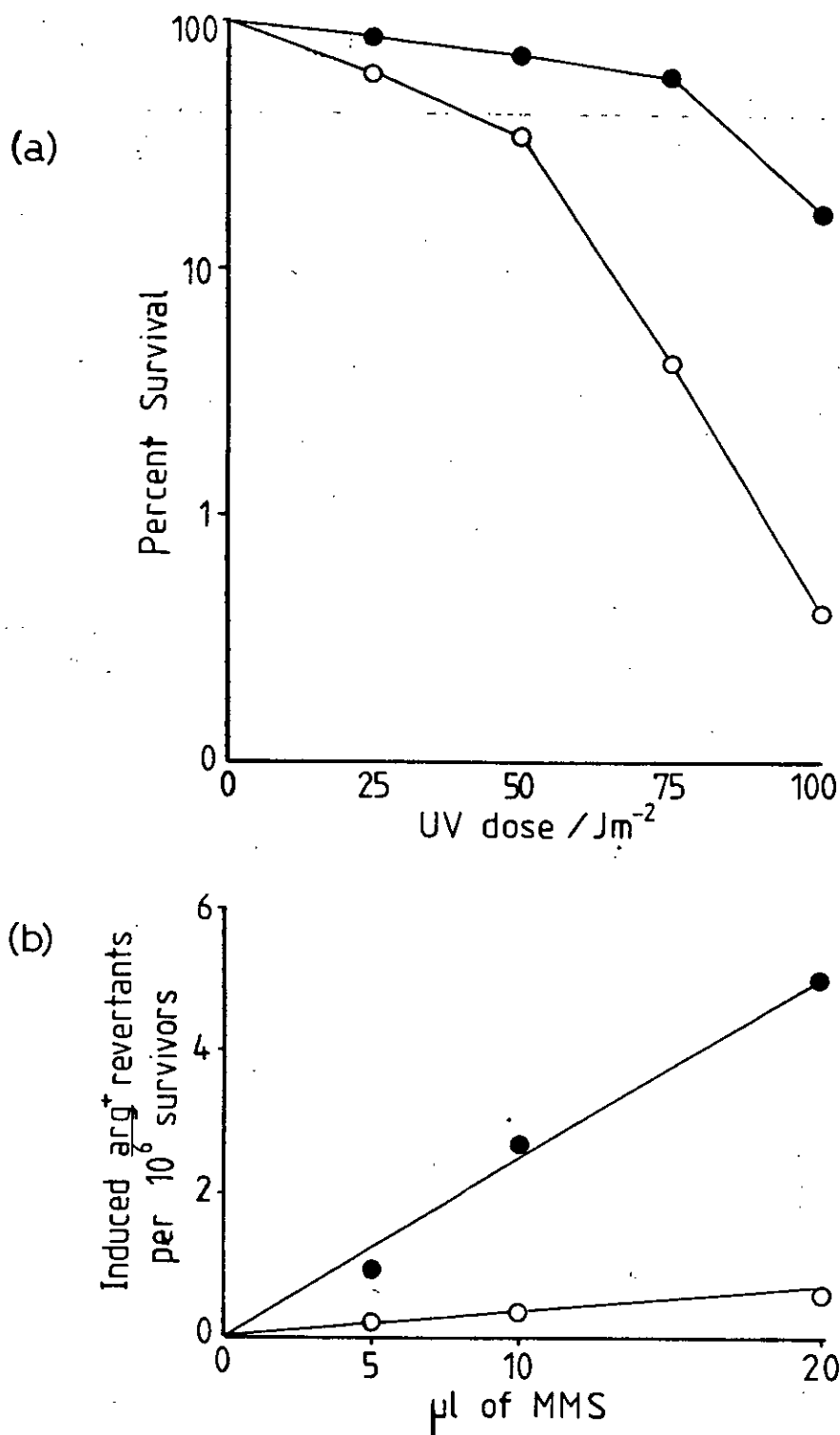


Fig. 3.1

Two aspects of the Uvp phenotypes of R46:

(a) Ultraviolet dosage-survival curves and (b) MMS-induced mutagenesis of ●, AB1157 (R46); ○, AB1157.

See Chapter 2.e for details.

by both R46 and a deletion derivative of R46 called pKM101 (Mortelmans and Stocker, 1979). In addition to protecting against UV and other DNA damaging agents (Drabble and Stocker, 1968; Mortelmans and Stocker, 1976; Tweats *et al*, 1976; Walker, 1977; Mortelmans and Stocker, 1979), R46 and pKM101 cause enhanced UV-induced and chemical mutagenesis (McCann *et al*, 1975a; Mortelmans and Stocker, 1976; Walker, 1977; Oliver and Stacey, 1977; Mortelmans and Stocker, 1979; see Fig.3.1b) and an increased spontaneous mutation rate (McCann *et al*, 1975a; Mortelmans and Stocker, 1976; Walker, 1977). The spontaneous reversion rate of all types of base pair substitutions is enhanced by pKM101, but the reversion of insertion or deletion frameshift mutations is unaffected (Fowler *et al*, 1979). The plasmids increase the propagation and mutation frequency of UV-irradiated phage in unirradiated cells, a property called 'Plasmid-Mediated Reactivation' (Mortelmans and Stocker, 1976; Walker, 1977), and pKM101 has also been shown to increase the extent of 'Weigle'* reactivation and mutagenesis of UV-irradiated phage in irradiated cells (Walker, 1977, 1978b). All these effects constitute Uvp phenotypes. N3 also protects cells against the lethal effects of UV and confers an increase in methyl methanesulphonate (MMS)-induced mutagenesis (Babudri and Monti-Bragadin, 1977; Molina *et al*, 1979), but the Uvp phenotypes of this plasmid have not been as fully characterised as those of R46 and pKM101.

McCann *et al* (1975a) found a two-fold higher level of MMS-induced mutagenesis in S.typhimurium containing pKM101 than in strains containing R46. This is the only report of a difference in the Uvp phenotypes of these two plasmids, and no such difference

* see Witkin (1976)

is apparent from the UV-mutagenesis data of Mortelmans and Stocker (1979). The ability of pKM101 to enhance chemical mutagenesis has led to the incorporation of this plasmid into the Ames test for the detection of carcinogens as mutagens (Ames et al, 1973, 1975; McCann et al, 1975a). The plasmid renders the Salmonella tester strains more susceptible to mutagenesis by a wide variety of compounds, and allows several known carcinogens to be detected as mutagens, that were not recognisably mutagenic in the plasmid-free strains (McCann et al, 1975a,b).

By P22 transduction and conjugal transfer of R46 between strains of S.typhimurium, Mortelmans and Stocker (1979) obtained a number of deletion mutants of R46, in addition to pKM101. They provided tentative evidence for a class of deletion plasmids that had lost the UV-protection and UV-induced mutagenesis phenotypes but still conferred enhanced spontaneous mutagenesis. This implied that these Uvp phenotypes are determined by at least two functionally distinct genes. Mortelmans and Stocker correctly admit that this evidence is incomplete, and there is considerable contrary evidence suggesting that all the Uvp phenotypes are eliminated by inactivation of a single plasmid gene product. Walker (1978a) isolated at least four presumptive non-polar pKM101 point mutants deficient in their ability to enhance MMS-induced mutagenesis, and found that they had simultaneously lost ability to increase UV-survival, spontaneous mutagenesis, plasmid-mediated reactivation, and Weigle-reactivation. Moreover, Shanabruch and Walker (1980) screened 645 insertions of the transposon Tn5 into pKM101 and found that the 20 unable to increase UV- or MMS-induced mutagenesis had also lost all the other Uvp phenotypes described above.

Since these 20 Tn5 insertions were in at least 18 different locations throughout a 1.9 kb segment of the plasmid, and flanking insertions 2.4 kb apart had no effect on mutagenesis and repair (Shanabruch and Walker, 1980; Langer et al, 1981), it is unlikely that the loss of all these phenotypes could be explained by polar effects of the transposons on two or more functionally distinct genes. The Tn5 insertions localise a region of between 1.9 kb and 2.4 kb in length which is necessary for the Uvp phenotypes, while data presented in Chapter 6 (6.b.iii) show that a segment of 3.1 kb of R46 is sufficient to provide a Uvp⁺ phenotype. The above results strongly suggest either that Uvp is mediated by a single plasmid gene product, or that if more than one gene is involved then mutations in any one of them will result in the loss of all Uvp phenotypes. The genetic determinant of the Uvp phenotypes of pKM101 has been named muc (Shanabruch and Walker, 1980).

Studies of the expression of Uvp phenotypes in cells carrying various chromosomal mutations have offered insights into the possible mechanism of R46-mediated repair and mutagenesis.

Plasmid-mediated Uvp phenotypes are abolished in recA or lexA mutant hosts (Tweats et al, 1976; Walker, 1977; Mortelmans and Stocker, 1979), although the extent of loss of plasmid-mediated mutagenesis seems to depend on the particular lexA allele used (Monti-Bragadin et al, 1976; Walker, 1977; Waleh and Stocker, 1979; Todd et al, 1979). In contrast the Uvp phenotypes are independent of recB, recC, recF, polA, uvrA, uvrB, uvrC, uvrD and uvrF genotypes (McCann et al, 1975a; Tweats et al, 1976; Walker, 1977; Mortelmans and Stocker, 1979; Goze and Devoret, 1979).

The abolition of Uvp phenotypes by recA and lexA mutations suggests

a relationship with the host cell's 'SOS' system, a co-ordinated set of responses induced by DNA damage, that includes cell filamentation, λ prophage induction, and an inducible error-prone repair function called 'SOS repair' (Witkin, 1976). None of the SOS functions are expressed in recA or lexA mutants and the SOS system is believed to be induced via the activation of the protease activity of RecA protein. DNA damage induces this activity in the small amount of RecA normally present, resulting in proteolysis of LexA protein, the repressor of the recA gene. An increased quantity of proteolytic RecA is thus produced, and induces SOS responses by cleaving repressors of the relevant genes, and the repressors of certain prophages. lexA mutants are dominant mutations that produce a protease-resistant repressor and so prevent induction of recA and other SOS functions (for review see Gottesman, 1981).

R46 and pKM101 do not exert their effects by causing a general induction of SOS functions since the level of RecA protein (Little and Hanawalt, 1977; Walker and Dobson, 1979) and the frequency of induction of λ prophage (Goze and Devoret, 1979) are unaffected by the presence of pKM101. Instead, the plasmids appear specifically to increase the cell's capacity to carry out error-prone repair. This interpretation is supported by the synergistic effect on mutagenesis observed between pKM101 and a tif sfiA mutation at 42° (Walker, 1977): at this temperature such a mutant expresses SOS repair activity constitutively. Furthermore, the ability of pKM101 to complement chromosomal umuC mutants (Walker and Dobson, 1979) is most significant. umuC mutants are specifically deficient in host-coded error-prone repair activity, but retain other recA⁺ lexA⁺

dependent functions (Kato and Shionura, 1977; Kato et al, 1977; Walker and Dobson, 1979). The addition of pKM101 to a umuC⁻ strain restores mutagenesis and repair to a level considerably higher than that observed in a plasmid-free umuC⁺ strain (Walker and Dobson, 1979).

The simplest interpretation of these results is that the plasmid muc gene product is functionally analogous to that of the chromosomal umuC gene (Goze and Devoret, 1979; Walker and Dobson, 1979), although this must be reconciled with the observation that a umuC⁻ mutant is no more sensitive to killing by UV-irradiation than a umuC⁺ strain (Walker and Dobson, 1979). The Uvp phenotypes may result from the activity of a plasmid-coded error-prone polymerase or a factor which makes an existing host polymerase error-prone (Walker, 1977). McPhee (1974) reported a polymerase activity associated with another UV-mutagenesis enhancing IncN plasmid, R205 (= R-Utrecht), in S.typhimurium, polA⁻, and Ando and Arai (1981) have recently found a similar activity determined by N3 in E.coli. Errors in repair mediated by such a polymerase might result from the absence of 3'→5' proof-reading activity (Radman et al, 1977), or from the ability to 'by-pass' pyrimidine dimers in the template DNA strand by addition of random nucleotides opposite the lesion. However, Kromish and Walker (1979) were unable to detect polymerase activity determined by pKM101 or by R205 in an E.coli polA⁻ strain.

Tweats et al (1976) demonstrated that following UV-irradiation, protein synthesis is required to obtain the UV-protective effect of R46, suggesting an inducible process. Furthermore enhanced reactivation of irradiated phage in pKM101-containing cells is considerably increased by prior irradiation of the cells with UV

(Walker, 1977). These results strongly imply that the plasmid-coded repair function is at least partly inducible by UV. Plasmid-mediated reactivation of phage also occurs in unirradiated cells, however, in which the cell's error-prone repair system (umuC product) is not induced (Walker, 1977; Walker and Dobson, 1979). This suggests either that the plasmid muc gene is more sensitive than umuC to induction mediated by damaged DNA, or that a residual level of plasmid-coded repair activity is expressed constitutively. A basal level of uninduced muc gene expression could also account for the enhanced spontaneous mutagenesis determined by R46 and pKM101.

A possible model for control of expression of the Uvp phenotypes is that the plasmid muc gene is partially or largely repressed by the lexA⁺ gene product, and that full induction occurs via proteolytic cleavage of this repressor by RecA protease under conditions of SOS induction. This model suggests that the Uvp phenotypes of R46 are a plasmid-borne component of the SOS response. Recent results using muc-lac fusion plasmids support the predictions of this model; β -galactosidase is produced constitutively by such plasmids in an unirradiated spr (= lexA⁰) recA mutant but not in an unirradiated wild-type strain (G. Walker, personal communication).

(c) Conjugal Transfer of R46 and N3

(i) Frequencies of transfer in broth and on solid media

IncN plasmid conjugal transfer, N pili, and IncN donor-specific phages have been discussed briefly in Chapter 1. Early measurements of transfer frequencies of R46 and N3 (e.g. Datta and Hedges, 1971) were performed in liquid cultures under conditions similar to those employed for F-like plasmids, and showed a poor efficiency of

transfer (typically 10^{-5} - 10^{-3} transconjugants per donor cell) relative to F transfer (typically 1 transconjugant per donor). Dennison and Baumberg (1975), however, demonstrated that IncN plasmid transfer was enhanced by a delayed transconjugant selection technique that allowed mating to occur on a solid medium. They concluded that IncN plasmids were de-repressed for transfer functions and that the low transfer frequencies previously observed were due to inefficient mating pair formation in liquid media. Indeed, when mating was performed on agar plates, prior incubation of the mixed parental cultures in broth gave no significant increase in transconjugant formation, suggesting that virtually all the mating pair formation occurred on the solid medium. These conclusions are supported by the apparently fragile nature of N pili (Bradley, 1979). These are easily detached from cells in liquid and may thus mediate mating pair formation more efficiently on solid surfaces, where shearing forces are reduced.

The transfer frequencies reported by Dennison and Baumberg for R46 and an N3 derivative, although improved by the plate mating method, were only approximately 10^{-3} per donor. However, when donor strains containing these plasmids are mixed with recipients on membrane filters and allowed to mate for 30 min on a nutrient agar surface, transconjugant frequencies of over 100% per donor are observed. Table 3.1 shows comparative data from broth matings and filter matings (see Chapter 2.c) for R46, N3 and a third IncN plasmid R390.

Table 3.1 Transfer frequencies of IncN plasmids

<u>Plasmid</u>	<u>Frequency of Transfer^a</u>	
	<u>Broth mating</u>	<u>Filter mating</u>
R46	4×10^{-4}	2
N3	3×10^{-4}	4
R390	4×10^{-4}	6.5×10^{-1}

^a Frequency of transfer = mean number of transconjugants per donor cell from two or more experiments. Donor strains were ED395 and recipients ED3818.

These results demonstrate that in favourable mating conditions the transfer of R46 and N3 is at least as efficient as that reported for any other conjugative plasmid. This strongly supports the view that IncN plasmid transfer functions are de-repressed.

Bradley et al (1980) have shown that the transfer frequencies of plasmids in a number of other incompatibility groups is similarly enhanced by a surface mating technique, and the majority of such plasmids appear to determine constitutive synthesis of conjugation pili.

The relatively low transfer efficiencies observed by Dennison and Baumberg (1975) in plate matings may have been partly due to the presence of nalidixic acid, a known inhibitor of plasmid DNA transfer (Barbour, 1967). Burman (1977) reported that transfer of N3 and other IncN plasmids is resistant to nalidixic acid, but his data were derived from mating mixtures in broth (with and without Nal) which were all subsequently plated on agar containing the drug. If no significant mating pair formation occurred in the broth, all plasmid transfers would have been subject to Nal-inhibition. Preliminary results from membrane filter matings on

Table 3.2 F-mediated enhancement of N3 transfer in broth matings

Plasmid(s) in ED395 donor	Plasmid in ED3818 recipient:		Resulting F-mediated mating aggregates	Frequency of transfer of N3 ¹	Fertility enhancement Index ²
	Plasmid	Genotype			
N3	-	-	none	3×10^{-4}	1
N3, JCFL0 (Flac)	-	-	stable	3×10^{-2}	100
N3, EDFL171	-	-	stable	5×10^{-2}	170
N3	JCFL0	Flac	stable	9×10^{-2}	300
N3	EDFL171	Flac traΔI ⁻	stable	9×10^{-2}	300
N3	JCFL83	Flac traD	stable	3×10^{-2}	100
N3	JCFL102	Flac traM	stable	1×10^{-1}	500
N3	JCFLG42	Flac traG42 ³	unstable	1.5×10^{-2}	50
N3	JCFLG79	Flac traG79 ³	unstable	1×10^{-2}	30
N3	KPF48	Flac traN	unstable	5×10^{-4}	2
N3	JCFL1	Flac traA	none	3.5×10^{-4}	1
N3	JCFL81	Flac traG81 ³	none	4×10^{-4}	1

¹ Frequency of transfer of N3 = mean transconjugant frequency per donor in 30 min broth matings.

² Fertility enhancement is calculated relative to the transfer frequency of N3 in ED395(N3) x ED3818 crosses.

³ Cells containing Flac traG42 or traG79 are piliated, and are sensitive to male specific phages. Those containing Flac traG81 are non-piliated and phage resistant.

nutrient agar containing nalidixic acid indicate that the transfer frequency of N3 is reduced at least 10-fold by the presence of the drug (data not shown). Burman's conclusions may thus need to be re-evaluated.

(ii) Fertility enhancement of N3 by Flac

The plasmid Flac is efficient at forming stable mating aggregates between donor and recipient cells in liquid culture, and unlike N3 transfers at high frequencies in liquid mating experiments. If the poor transfer of IncN plasmids in liquid media is due, as suggested, to inefficient mating pair formation, the additional presence of Flac in donor cells might be expected to enhance the transfer efficiency of N3 in broth matings. This is true for an integrated F factor since Burman (1977) observed a >100-fold increase in N3 transfer frequency when Hfr strains were used as donors rather than F⁻ strains.

Table 3.2 shows the frequency of N3 transfer from ED395 (N3, Flac) was approximately 100 times greater than from ED395 (N3) without Flac present. The frequency of transfer of Flac (8.5×10^{-1}) was not affected by the presence of N3 in the donor strain. A similar enhancement of N3 transfer was caused by EDFL171, a deletion mutant of Flac completely deficient in transfer but determining normal F pili, showing that the transfer of F DNA is not required for this effect.

If Flac-mediated enhancement of N3 transfer is simply a consequence of efficient F mating-aggregate formation, it would also be expected to occur when Flac is present in the recipient strain only. Table 3.2 shows this to be the case. The effect was also observed using ED3818 (EDFL171) as recipient. In this cross

there could be no transfer of the Flac derivative into the ED395 (N3) donor cells to effect genetic complement in the formal sense.

The F transfer functions required for enhancement of N3 transfer were further investigated using a number of different Flac tra mutants in the ED3818 recipient strains. Table 3.2 shows that the Flac tra mutants in ED3818 that promote stable mating aggregate formation enhance the transfer frequency of N3 from ED395 (N3) by at least 100-fold. In contrast the non-piliated mutants, which give no F-mating aggregates, showed no such effect. The Flac traG42, traG79 and traN mutants constitute a third group with respect to mating aggregate formation. Cells containing any of these three mutants are piliated, but form only few mating aggregates with F⁻ cells, and these aggregates have thus been inferred to be unstable (Achtman and Skurray, 1977; Manning and Achtman, 1979). It is not clear, however, to what extent these mutations may affect the rate of initiation of mating aggregates rather than their stability once formed. Flac traG42 and traG79 mutants in ED3818 increased the frequency of N3 transfer from ED395 (N3) by only 30-50 fold, while Flac traN gave no significant increase at all (Table 3.2). This difference in behaviour between the traG and traN plasmids may conceivably reflect a difference in the rate and duration of aggregate formation determined by these mutants.

In summary, the enhanced transfer of N3 mediated by Flac appears to depend solely on Flac transfer genes concerned with the formation of mating aggregates. The physical proximity of cells in such aggregates probably facilitates the formation of N3-determined conjugation bridges, which would otherwise be formed

TABLE 3.3 Fertility inhibition of IncN, IncP and IncW plasmids:
data from broth matings

a) Transfer frequencies of plasmid A in the presence of plasmid B in the same donor strain (ED395). Recipients were ED3818.

<u>Plasmid A</u>	<u>Plasmid B</u>					
	<u>None</u>	<u>R46</u>	<u>N3</u>	<u>R390</u>	<u>RP1</u>	<u>Sa</u>
R46	4×10^{-4}	-	-	-	2×10^{-4}	NT
N3	3×10^{-4}	-	-	-	3×10^{-5}	$< 4 \times 10^{-7}$
R390	4×10^{-4}	-	-	-	2×10^{-4}	3×10^{-7}
RP1	3×10^{-5}	$< 3 \times 10^{-7}$	$< 10^{-6}$	$< 3 \times 10^{-8}$	-	6×10^{-5}
Sa	4×10^{-4}	NT	2×10^{-4}	8×10^{-4}	$< 4 \times 10^{-7}$	-

b) Fertility inhibition indices: transfer frequency of plasmid A in presence of plasmid B relative to uninhibited transfer of A.

<u>Plasmid A</u>	<u>Incompatibility Group</u>	<u>Plasmid B</u>				
		<u>R46</u>	<u>N3</u>	<u>R390</u>	<u>RP1</u>	<u>Sa</u>
R46	IncN	-	-	-	5×10^{-1}	NT
N3	IncN	-	-	-	10^{-1}	$< 1.5 \times 10^{-3}$
R390	IncN	-	-	-	5×10^{-1}	7.5×10^{-3}
RP1	IncP	$< 10^{-2}$	$< 3 \times 10^{-2}$	$< 10^{-3}$	-	2
Sa	IncW	NT	5×10^{-1}	2	$< 10^{-3}$	-

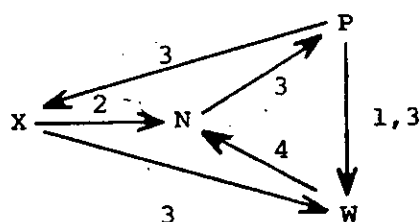
NT = not tested. '-' denotes incompatible plasmids

less efficiently in the liquid medium. These results are therefore consistent with the view that low frequencies of IncN plasmid transfer in broth are due to a deficiency in mating pair formation rather than repression of plasmid transfer functions.

(iii) Fertility inhibition

As discussed briefly in Chapter 1, fertility inhibition relationships are exhibited between plasmids of incompatibility groups N, P, W and X. These relationships are summarised in Fig. 3.3.

Fig. 3.3. Fertility inhibition relationships between plasmids of incompatibility groups N, P, W and X



1. Datta and Hedges, 1972

3. Olsen and Shipley, 1975

2. Pinney and Smith, 1974

4. This thesis

Arrows indicate the direction of inhibition.

Measurements of the transfer inhibition interactions between pairs of plasmids in three of these groups are shown in Table 3.3. These data, obtained from broth mating experiments, confirm the published results, and additionally demonstrate the inhibition of IncN plasmid transfer by an IncW plasmid. Since plasmid transfer frequencies are normally low in broth matings the transfer of plasmids suffering fertility inhibition in these experiments was usually undetectable, and only minimum estimates of the fertility inhibition indices could be obtained. The interaction between IncN

and IncP plasmids was investigated further, however, using the more efficient membrane filter mating technique. Table 3.4 shows that under these conditions IncN plasmids may reduce the transfer frequency of an IncP plasmid by factors of approximately 10^{-4} .

Table 3.4. Fertility inhibition of RP1 by IncN plasmids: data from filter matings

	Second plasmid present in donor			
	<u>none</u>	<u>R46</u>	<u>N3</u>	<u>R390</u>
Transfer frequency of RP1	5×10^{-1}	10^{-5}	6×10^{-5}	10^{-4}
Fertility inhibition index	-	2×10^{-5}	1.2×10^{-4}	2×10^{-4}

Donor strains were ED395 containing relevant pairs of plasmids.

Recipients were ED3818. The transfer of R46, N3, and R390

was not significantly inhibited by RP1.

CHAPTER 4. PHYSICAL CHARACTERISATION OF R46 AND N3

(a) Introduction

Physical maps of plasmid molecules can be constructed by locating the relative positions of restriction endonuclease cleavage sites on the plasmid DNA. For R46 and N3 such data serves two main purposes. Firstly it provides useful information for the construction of deletion and recombinant derivatives by in vitro recombination of restriction cleavage fragments. Secondly it facilitates the analysis of these and other derivatives, and allows their phenotypes to be correlated with defined segments of the parent molecules. The total size of a plasmid can be estimated by summing the sizes of restriction cleavage fragments derived from it, but a more accurate method is to determine the contour lengths of intact circular molecules under the electron microscope.

Electron microscopy can also be used to identify regions of DNA homology within or between plasmids. If denatured plasmid DNA is allowed to re-anneal briefly before spreading on electron microscope grids, single-stranded regions containing complementary base sequences can form double-stranded 'snapback' structures. These are indicative of homologous sequences in inverted orientation in the double-stranded DNA. If linear molecules, produced by restriction cleavage, are used in such experiments, the positions of the 'snapback' structures can be determined in relation to restriction enzyme sites, and hence the inverted repeat sequences located on the physical map of the plasmid.

(b) Sizes of R46 and N3 molecules

The sizes of R46 and N3 were determined by DNA contour length

Table 4.2 Sizes of Restriction Endonuclease Cleavage Fragments of R46

<u>Fragment</u>	<u>Restriction Endonuclease</u>								
	<u>EcoRI</u>	<u>HindIII</u>	<u>BglII</u>	<u>PstI</u>	<u>SalI</u>	<u>BamHI</u>	<u>KpnI</u>	<u>SmaI</u>	<u>XhoI</u>
A	36.6	47.4	17.1	36.5	21.65	42.65	40.9	35.75	51.7
B	7.5	2.38	8.5	4.75	10.0	5.95	6.1	15.95	
C	5.5	1.84	6.0	2.5	8.3	3.1	4.7		
D	1.65	0.16	5.7	2.4	2.25				
E	0.45		4.75	1.5	0.57				
F			4.6	1.2	0.53				
G			3.85	1.0					
H			0.87	0.6					
I			0.35	0.45					
J				0.3					
K				0.25					
L				0.25					

Sizes are given in kilobase pairs. Sizes of fragments over 20 kb were calculated by subtraction from the total size of 51.7 kb.

measurements in the electron microscope. R46 and N3 plasmid DNA preparations contained a sufficient proportion of nicked open circular molecules suitable for such measurements. These were spread and photographed, and their contour lengths determined. Similar measurements were made of double-stranded ϕ X174 molecules on the same grid to provide size standards. Comparisons of the values obtained then allowed the sizes of R46 and N3 to be calculated (see Table 4.1).

Table 4.1. Sizes of R46 and N3 determined by electron microscopy

<u>Plasmid</u>	<u>Number of molecules measured</u>	<u>Mean size/kb</u>	<u>Standard error/kb</u>
R46	11	51.7	0.2
N3	19	57.7	0.3

Estimates of the total sizes of R46 and N3 were also obtained by summing the sizes of BglIII + KpnI restriction enzyme double-digest fragments, determined from their mobilities in agarose gel electrophoresis (see below). The values obtained were 52.2 kb and 58.1 kb respectively, which agree well with those shown in Table 4.1. The sizes determined from electron microscopy are adopted here as the best estimates of total plasmid length.

(c) Physical Characterisation of R46

(i) Construction of the restriction endonuclease cleavage map

The sizes of restriction fragments of R46 DNA produced by the enzymes EcoRI, HindIII, BglIII, PstI, SalI, BamHI, KpnI, SmaI and XhoI were estimated from their mobilities in agarose gel electrophoresis, with reference to λ DNA fragments of known size (Table 4.2, Fig. 4.1, Fig. 4.4). Sizes of double-digestion fragments produced

by pairs of restriction enzymes were similarly determined (examples of such digests are shown in Fig. 4.1). Comparisons of the sizes of double-digest fragments with those produced by each enzyme individually permitted the relative map positions of most cleavage fragments to be deduced. For some fragments it proved necessary to supplement the double-digest data with information derived from cleavage of R46 deletion plasmids, and of recombinant clones containing specific R46 restriction fragments (described in Chapter 5). For example, the order of fragments BglIII-I, E, C and F was determined by BglIII digestion of a series of deletion plasmids of decreasing size, pED940-pED946 (Ch. 5.b.ii).

Restriction fragments of less than 0.3 kb would not usually have been detected on the agarose gels used in these experiments. However, EcoRI and HindIII digests (and double-digests with these enzymes) were also analysed by polyacrylamide gel electrophoresis of [^{32} P]-end-labelled restriction fragments (see Fig. 4.1). This technique allows the detection of fragments down to about 30 base pairs.

The restriction cleavage map of R46 is shown in Fig. 4.2. If restriction enzyme recognition sequences were distributed randomly, any one hexanucleotide-specific enzyme would cleave DNA on average every 4096 base pairs. It is striking that a large region of the R46 map contains many fewer cleavage sites than would be predicted on a random basis, and that the majority of sites are located within approximately one third of the molecule. The possible significance of this phenomenon (also observed for N3, see below) is discussed in Chapter 6. Of the nine hexanucleotide-specific restriction enzymes used, seven produce a cleavage fragment of >35kb, too large

Fig. 4.1

- (a) Restriction endonuclease cleavage patterns of R46 DNA obtained by electrophoresis in 0.7% agarose gels. Examples of double enzyme digestion data used for mapping the restriction sites.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>	<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>
1	λ 7	<u>HindIII</u>	12	R46	<u>BamHI</u>
2	R46	<u>EcoRI</u>	13	R46	<u>BamHI</u> + <u>PstI</u>
3	R46	<u>EcoRI</u> + <u>BglII</u>	14	R46	<u>PstI</u>
4	R46	<u>BglII</u>	15	λ 7	<u>HindIII</u>
5	R46	<u>BglII</u>	16	R46	<u>EcoRI</u>
6	R46	<u>BglII</u> + <u>PstI</u>	17	R46	<u>EcoRI</u> + <u>KpnI</u>
7	R46	<u>PstI</u>	18	R46	<u>KpnI</u>
8	λ 7	<u>HindIII</u>	19	λ 7	<u>HindIII</u>
9	R46	<u>SalI</u>	20	λ 7	<u>HindIII</u>
10	R46	<u>SalI</u> + <u>HindIII</u>	21	R46	<u>EcoRI</u>
11	R46	<u>HindIII</u>	22	R46	<u>EcoRI</u> + <u>HindIII</u>
			23	R46	<u>HindIII</u>

Sizes of HindIII fragments of λ 7 DNA are marked in kilobases.

- (b) Autoradiogram of [^{32} P]-end-labelled R46 restriction fragments after 8% polyacrylamide gel electrophoresis.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>
1	R46	<u>EcoRI</u>
2	R46	<u>EcoRI</u> + <u>HindIII</u>
3	R46	<u>HindIII</u>

Sizes of fragments are marked in base pairs, and were estimated by comparison with unlabelled fragments of known size on the same gel.

Fig. 4.1

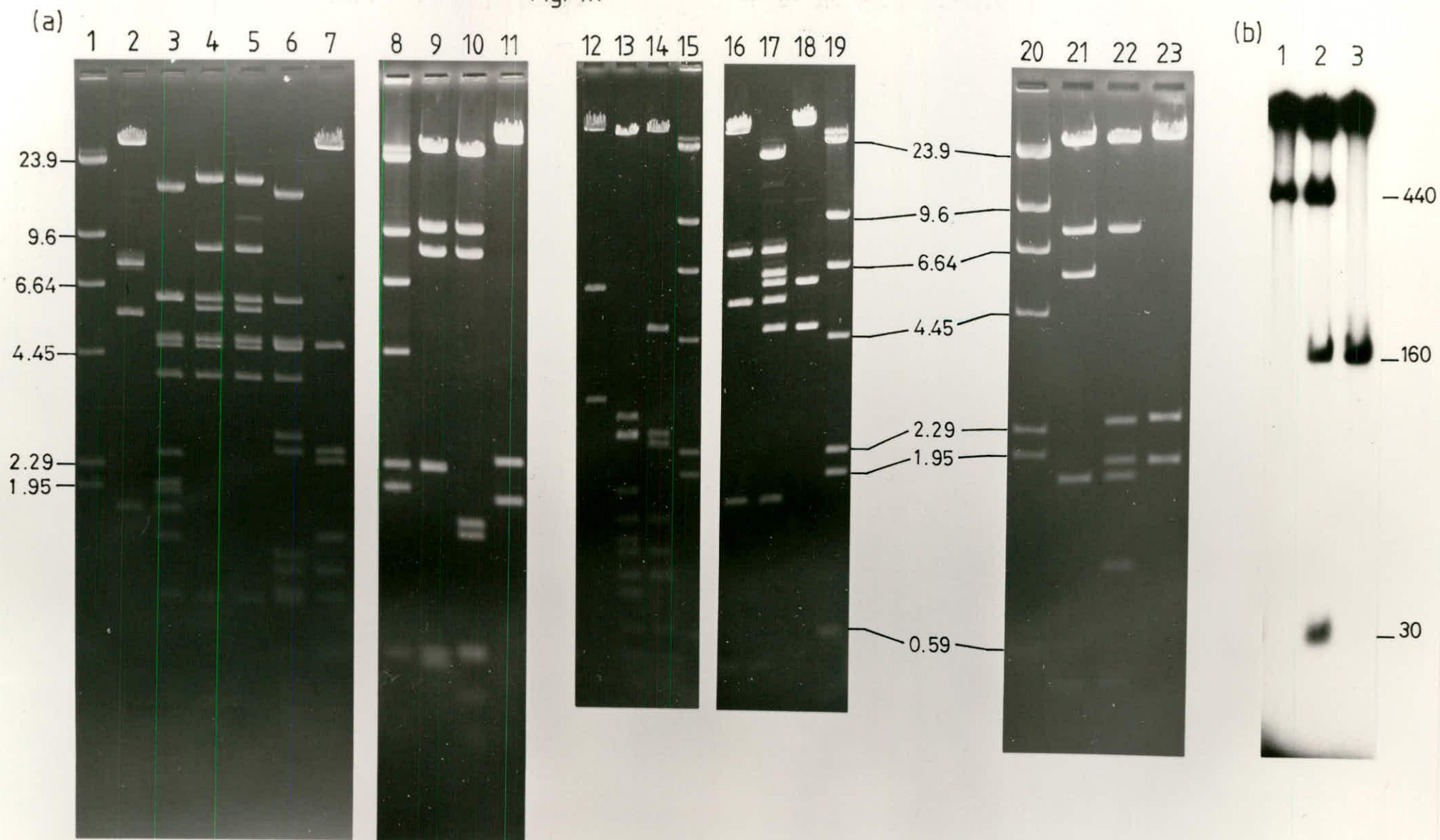
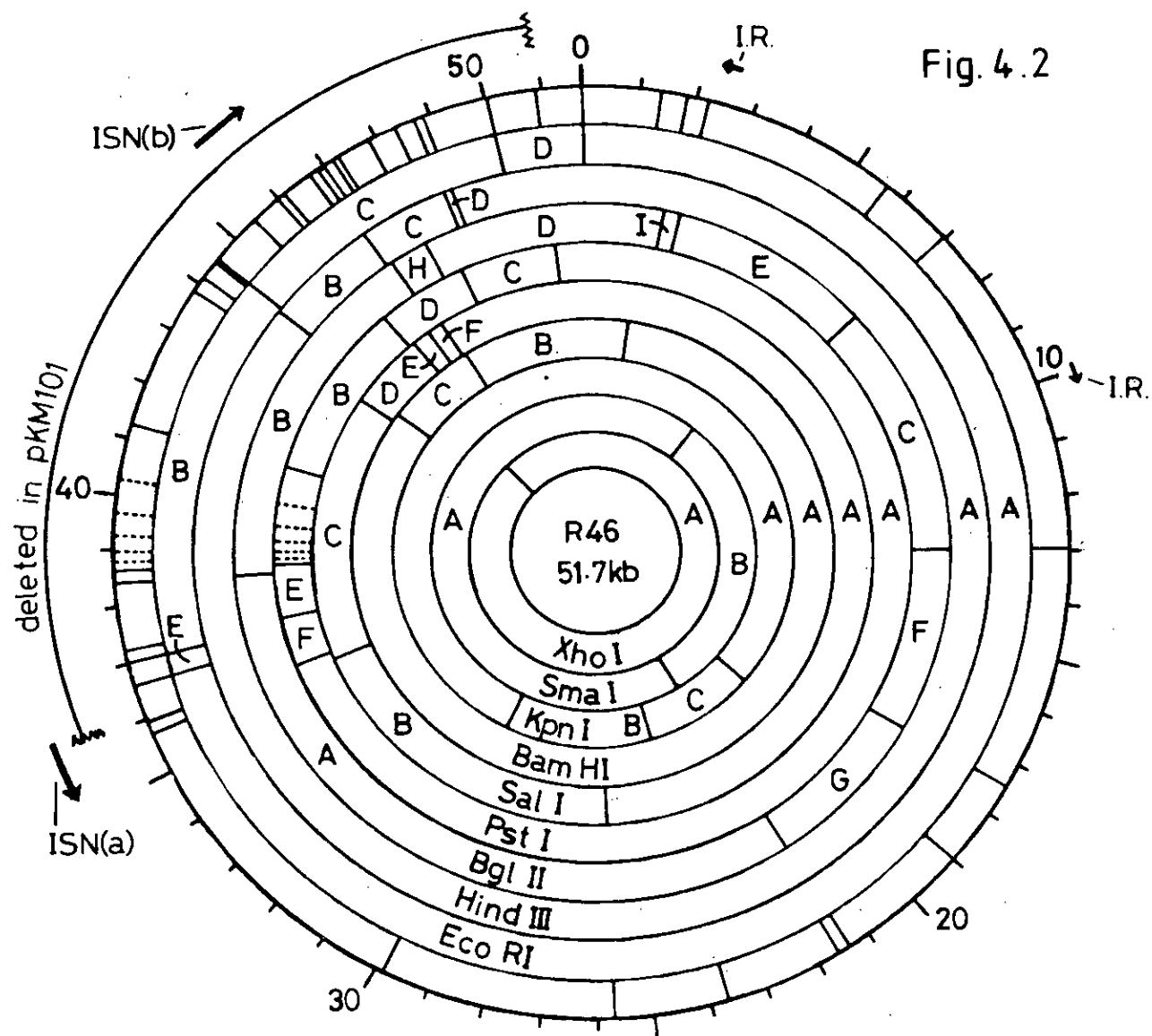


Fig. 4.2. Restriction endonuclease cleavage map of R46 for the enzymes EcoRI, HindIII, BglIII, PstI, SalI, BamHI, KpnI, SmaI, and XhoI. The outer circle shows the sites for all nine enzymes. Fragments produced by each enzyme are labelled alphabetically in order of decreasing size (see Table 4.2). Dotted lines indicate the approximately location of six small PstI fragments, the order of which was not determined. The R46 DNA deleted in pKM101 is marked. The positions of the short inverted repeats (I.R.) and the ISN inverted repeats are those determined by electron microscopy (see Fig. 4.3). Co-ordinates are marked in kilobases.

Fig. 4.2



for accurate size estimation using agarose gels. Only BglII has a fairly evenly distributed series of target sites (Fig. 4.2).

(ii) Location of the region of R46 deleted in pKM101

pKM101 is an Ap^{R} Uvp^+ Tra^+ deletion derivative of R46 that is included in bacterial strains used for the detection of carcinogens as mutagens, and has been used in several studies of the Uvp phenotypes (Mortelmans and Stocker, 1979; see Ch. 3.b.v.). Comparison of restriction digest products of R46 with those of pKM101 showed that all the fragments absent from pKM101 are contiguous in R46. This suggests that the two plasmids differ by deletion of a single region of R46 DNA. pKM101 contains single cleavage fragments for EcoRI, BamHI and PstI (although additional fragments of <0.3 kb would not have been detected), and two cleavage fragments for SalI, one of which co-migrates with SalI-B of R46 on agarose gels. These data (together with others not shown) allowed the end-points of the deletion to be deduced. They lie between SalI (co-ordinate 35.8 kb) and EcoRI (36.6 kb) at one end, and between two EcoRI sites (50.05 and 51.7 kb) at the other (see Fig. 4.2). Moreover, each end-point lies close to one of the PstI sites at R46 co-ordinates 35.95 and 50.9 kb, respectively (Fig. 4.2). At least one of these PstI sites is retained in pKM101, and if both are present they lie less than 0.3 kb apart.

(iii) Self-annealing of R46 DNA

R46 molecules were made linear by digestion with XhoI, an enzyme having a single cleavage site in R46 DNA (Fig. 4.2). The molecules were denatured, allowed to re-anneal briefly, and spread for examination and photography under the electron microscope. Double-stranded ϕX174 DNA and single-stranded M13 molecules were included on the same grid as size standards; this allowed calculation of the lengths of double- and single-stranded regions of self-annealed R46 DNA.

Fig. 4.3 Electron micrograph of self-annealed R46 DNA

Double-stranded regions, C and E, are indicated by the thicker lines in the diagram. A single-stranded M13 size standard is marked. Double-stranded ϕ X174 standards were photographed in an adjacent region of the grid. A fragment of single-stranded DNA is visible at the left of the photograph, partly overlapping region G.

The inverted repeats in region C are referred to in the text as 'ISN'.

Length estimates, derived from measurements of 8 molecules:

<u>Region</u>	<u>Mean length/kb</u>	<u>Std. error/kb</u>
A	0.06	0.02
B	9.4	0.3
C	0.865	0.03
D	7.6	0.3
E	0.34	0.02
F	7.3	0.26
G	23.8	1.1

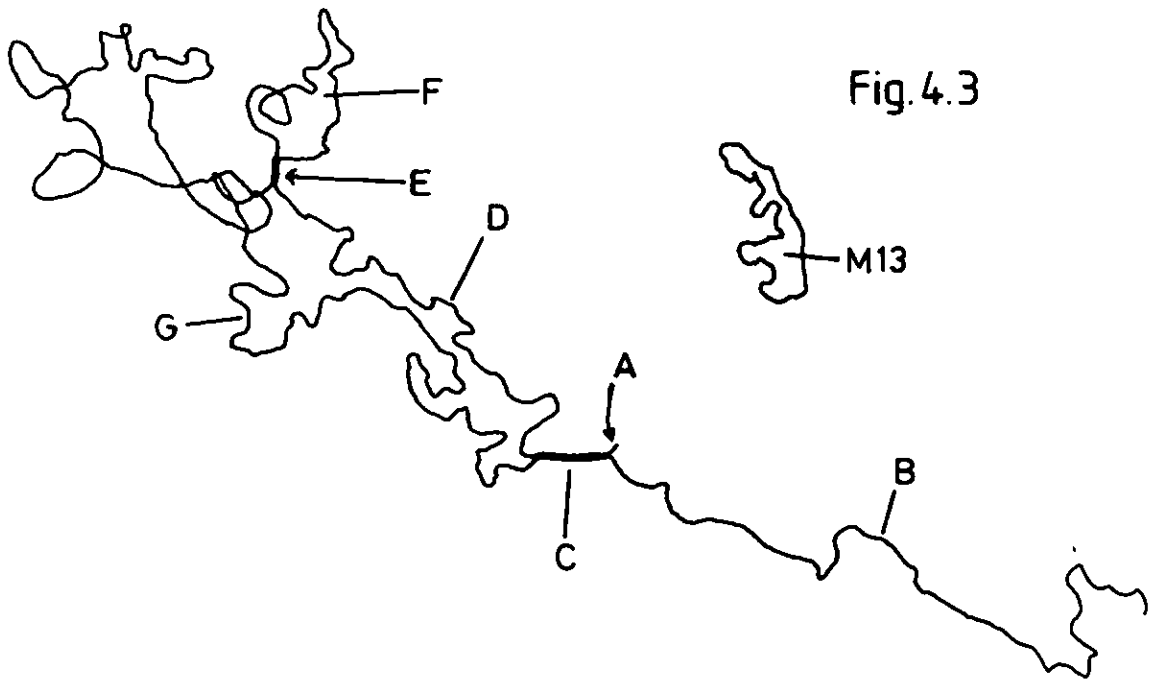
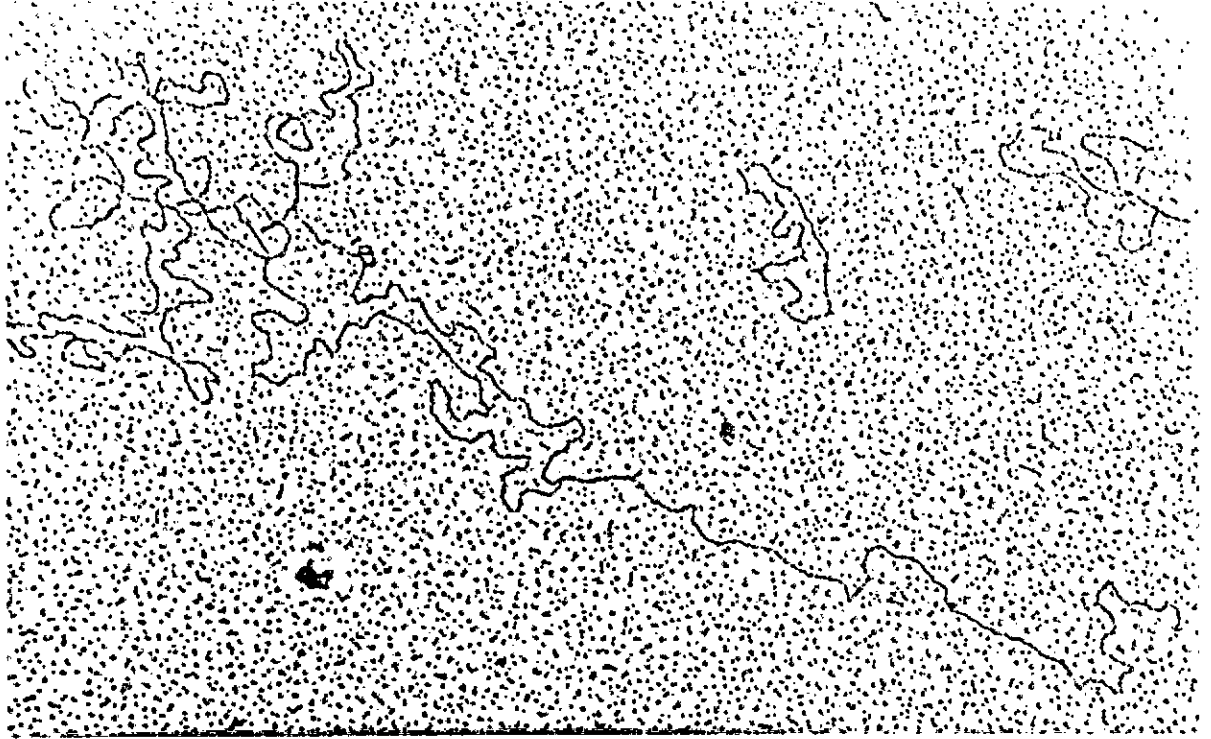


Fig.4.3

The structures observed in these experiments (an example is shown in Fig. 4.3) indicate that R46 contains two pairs of inverted repeat sequences. One pair of self-annealed sequences forms a double-stranded stem of 340 bp (± 15 bp) with an intervening single-stranded loop of 7.3 kb (± 0.26 kb) (E and F in Fig. 4.3). The dimensions of this stem-loop structure are similar to that observed on pKM101 by Langer *et al* (1981) and the two structures lie in equivalent positions on the R46 and pKM101 maps. This pair of short repeats flanks the Uvp determinant of R46 and possibly also the plasmid replication region (Langer *et al*, 1981; Chapter 6).

The second pair of inverted repeats on R46 forms a double-stranded region of 865 bp (± 30 bp) (C in Fig. 4.3). These sequences will be referred to as 'ISN(a)' and 'ISN(b)' for clarity, and for reasons that will become evident below. The XhoI cleavage site lies within the shorter intervening segment between this pair of sequences, since single-stranded linear tails are formed instead of a loop in the self-annealed molecules (A and B in Fig. 4.3). The lengths of these single-stranded tails indicate that the two copies of ISN on R46 are separated by approximately 9.5 kb, and one copy must lie extremely close to the XhoI restriction site since one of the tails is very short (≤ 100 bp). The positions of both pairs of inverted repeats, determined from electron microscopy, are indicated on the restriction cleavage map of R46 (Fig. 4.2). Fig. 4.2 shows that each ISN repeat coincides with a pair of closely spaced cleavage sites for SalI and PstI, and that the two pairs of sites are in inverted repeat orientation. This suggests that the ISN repeated sequence may contain within it a SalI and a PstI restriction site.

- (iv) Evidence for inversion of a segment of R46 flanked by inverted repeats.

Langer and Walker (1981) have also measured the sizes of restriction endonuclease cleavage fragments of R46. The values they obtained, however, whilst in broad agreement with those in Table 4.1, differ strikingly in certain cases, indicating a significant difference between the physical organisation of the plasmid they examined and that of the R46 isolate employed here. These two forms of R46 will be referred to as R46B and R46A respectively.

The discrepancies in cleavage fragment sizes were confirmed when restriction digest products of R46A and R46B DNA were directly compared in agarose gel electrophoresis (see Fig. 4.4). For each enzyme used the total number of cleavage sites is the same in both forms of R46, and there is no evidence for a change in total plasmid size due to deletion or insertion. A comparison of the BglII digestion products of R46A and R46B shows that all the restriction fragments are common to both molecules, except two in each digest. The 17.1 kb and 8.5 kb BglII fragments of R46A are missing in R46B, which instead contains fragments of 22 kb and 3.6 kb not found in R46A. The sums of the sizes of these pairs of fragments are equal, suggesting that the difference in restriction patterns between R46A and R46B may be due to inversion of a DNA segment containing one or more BglII sites. A similar difference in restriction pattern is observed for the enzymes HindIII and BamHI (Fig. 4.4). The EcoRI digest products of R46A and R46B appear to differ by 0.75 kb in one fragment only (Fig. 4.4), but a similar size difference in the large EcoRI-A fragments of each plasmid would not be detectable on gels under the conditions used. Moreover these fragments must differ in length if the total plasmid size of R46A and R46B remains the same. In contrast the PstI, SalI, KpnI and SmaI digest patterns of R46B appear to be identical to those of R46A (Fig. 4.4, data not shown, data of Langer and Walker, 1981).

Fig. 4.4

0.7% agarose gel electrophoresis showing the difference in restriction cleavage patterns between R46A and R46B.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	λ 7	<u>HindIII</u>
2	R46A	<u>HindIII</u>
3	R46B	<u>HindIII</u>
4	R46A	<u>BamHI</u>
5	R46B	<u>BamHI</u>
6	λ 7	<u>HindIII</u>
7	R46A	<u>EcoRI</u>
8	R46B	<u>EcoRI</u>
9	R46A	<u>BglII</u>
10	R46B	<u>BglII</u>
11	R46A	<u>PstI</u>
12	R46B	<u>PstI</u>
13	λ 7	<u>HindIII</u>

Note that while the HindIII, BamHI, EcoRI and BglII restriction patterns differ between R46A and R46B, the patterns observed for PstI digestion are identical.

The sizes in kilobases of HindIII fragments of λ 7 DNA are indicated on the figure.

Fig. 4.4

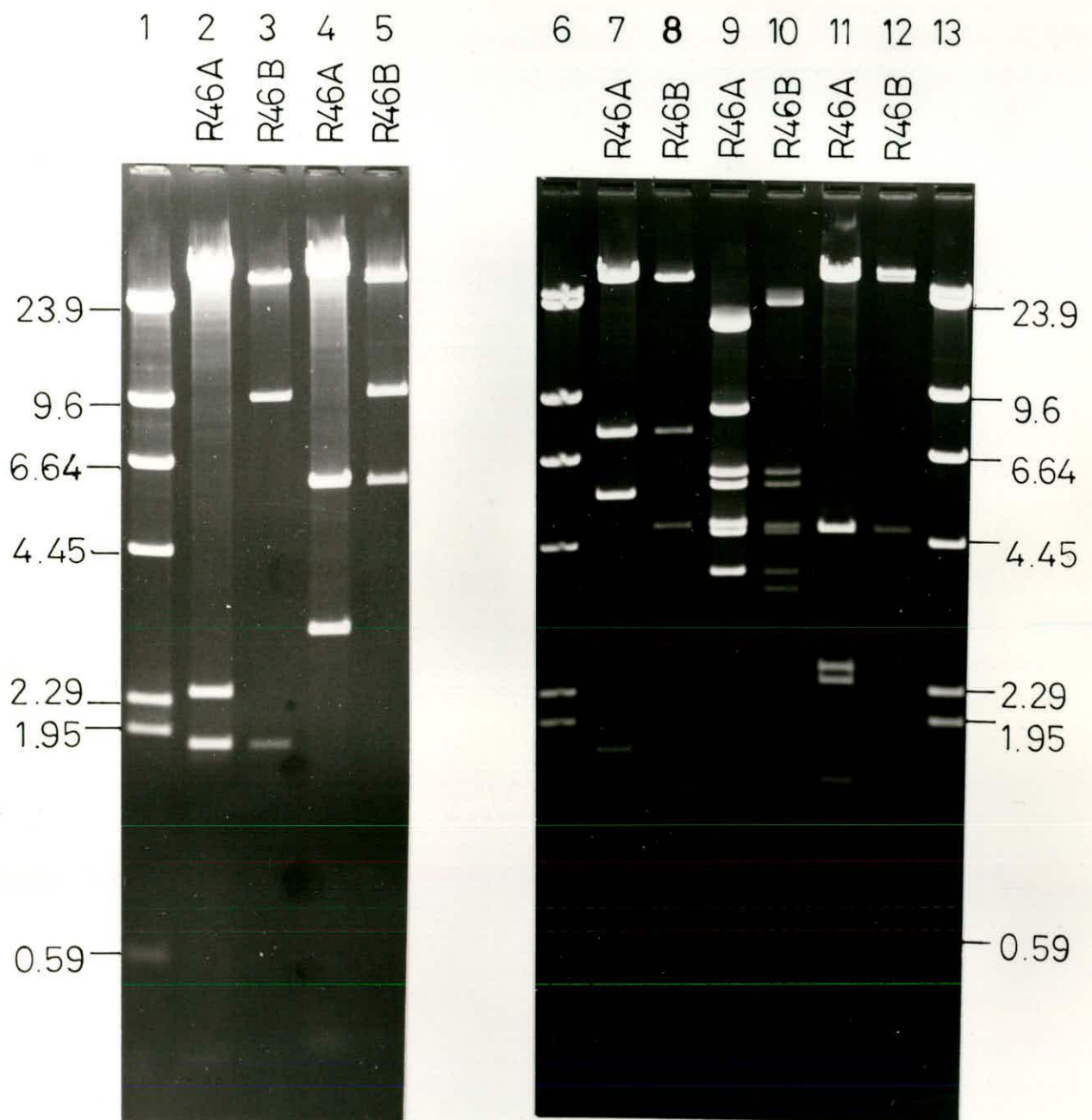


Fig. 4.5

Map of R46A showing the positions of the ISN inverted repeats, and restriction fragments the sizes of which are altered in R46B. For each restriction enzyme indicated only two cleavage fragments are altered in size, and each of these must contain one end-point of the inversion that distinguishes the two forms of R46. Note that every fragment marked contains one of the ISN elements, suggesting that the end-points of the inversion may lie within these sequences.

The lettering of fragments and co-ordinates of the map correspond to those shown in Fig. 4.2.

Fig. 4.5

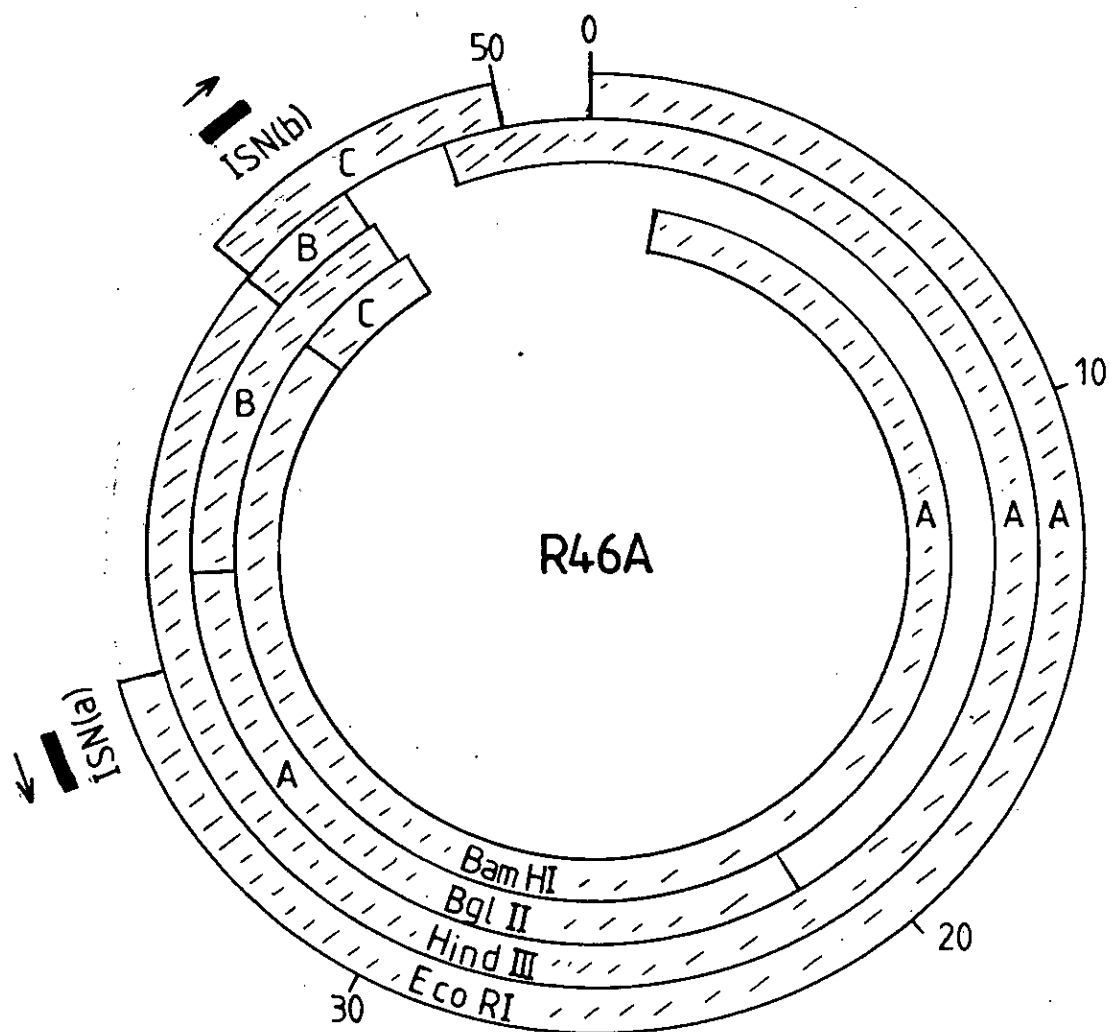


Table 4.3 Differences in restriction fragment sizes between
R46A and R46B

<u>Restriction fragment</u> ^c	<u>R46A</u>	<u>R46B (measured)</u> ^a	<u>R46B (predicted)</u> ^b
<u>EcoRI</u> -A	36.6	35-40	37.35
B	7.5	7.5	7.5
C	5.5	4.8	4.75
D	1.65	1.65	1.65
<u>HindIII</u> -A	47.4	<47 kb	40.3
B	2.4	9.6	9.5
C	1.85	1.85	1.85
<u>BglII</u> -A	17.1	22	22.05
B	8.5	6.0	6.0
C	6.0	5.7	5.7
D	5.7	4.75	4.75
E	4.75	4.6	4.6
F	4.6	3.85	3.85
G	3.85	3.45	3.55
<u>BamHI</u> -A	42.65	<42 kb	36.2
B	5.95	9.6	9.55
C	3.1	5.95	5.95

Sizes are in kilobase pairs.

- ^a Sizes of R46B fragments that do not co-migrate with R46A fragments in gel electrophoresis are approximate values based on single measurements only. EcoRI-A, HindIII-A and BamHI-A were too large for accurate size estimation under the gel conditions used.
- ^b Fragment sizes predicted for a plasmid derived from R46A by inversion of the segment between co-ordinates 35.8 and 46.1 kb.
- ^c Only certain fragments produced by each enzyme are listed. Those not shown are common to R46A and R46B.

Fig. 4.5 shows that all the restriction fragments that differ in size between the two forms of R46 contain one of the two copies of the 865 bp ISN inverted repeats described in section (c.iii) above. These repeated sequences constitute small regions of homology across which genetic recombination might occur, thereby causing inversion of the 9.5 kb intervening segment. This segment contains restriction sites for BglIII, HindIII, BamHI, EcoRI, SalI and PstI. If, as suggested above, the ISN elements each contain cleavage sites for SalI and PstI, such an inversion would leave the SalI and PstI restriction patterns unaltered, despite the presence of sites for these enzymes within the inverted region. Taking points between the SalI and PstI sites in each ISN repeat (co-ordinates 35.8 kb and 46.1 kb) as possible end-points of the proposed inversion, the intervening segment of the R46A restriction map was reversed, and the consequent changes in restriction fragment sizes calculated. Table 4.3 shows that this hypothetical inversion would change the restriction pattern of R46A to give cleavage fragments of sizes closely similar to those obtained from R46B. The two different forms of R46 probably do therefore differ by inversion of the DNA segment flanked by the two ISN inverted repeats, and these repeats most probably do contain cleavage sites for SalI and PstI.

(d) Physical Characterisation of N3

A restriction endonuclease cleavage map of N3 was constructed by methods similar to those used for R46. The sizes of N3 DNA cleavage fragments produced by eight different hexanucleotide-specific enzymes are given in Table 4.4. The relative positions of the majority of cleavage sites were determined from double enzyme digestion data and the resulting map is shown in Fig. 4.6. As with R46 a large region of N3 contains many fewer restriction sites than would be expected in a random DNA sequence of the same length, and the majority of sites lie within approximately one third



Table 4.4 Sizes of Restriction Endonuclease Fragments of N3

<u>Fragment</u>	<u>Restriction Endonuclease</u>							
	<u>EcoRI</u>	<u>HindIII</u>	<u>BglIII</u>	<u>PstI</u>	<u>SalI</u>	<u>BamHI</u>	<u>KpnI</u>	<u>SmaI</u>
A	48.2	53.1	14.0	42	25.3	40	31	57.15
B	8.3	2.0	11.4	4.75	13	7.8	18	0.55
C	0.6	1.45	8.3	3.9	8.2	4.2	4.6	
D	0.6	0.55	7.7	3.7	5.65	3.9	4.0	
E		0.45	7.3	2.25	2.1	1.9		
F		0.16	6.5	0.6	1.9			
G			1.55	0.55	0.9			
H			0.95		0.7			

Sizes are in kilobase pairs.

Sizes of fragments over 20 kb were calculated by subtraction from the total size of 57.7 kb.

Fig. 4.6

Restriction endonuclease cleavage map of N3 for the enzymes EcoRI, HindIII, BglII, PstI, SalI, BamHI, KpnI and SmaI. The outer circle shows the sites for all eight enzymes. Fragments produced by each enzyme are labelled alphabetically in order of decreasing size. Fragments smaller than 0.6 kb are not labelled. Arrows represent the possible locations of two copies of the ISN element, suggested by the presence of two pairs of closely spaced PstI and SalI sites. Co-ordinates are marked every 2 kb.

[illegible]

of the molecule. BglIII is again the only enzyme used which cleaves the plasmid at a widely distributed series of sites.

(e) Discussion

Although pKM101 was derived from R46 by two consecutive deletion events (Mortelmans and Stocker, 1979), the plasmid differs from R46 by the absence of a single contiguous DNA segment of approximately 15 kb. A similar conclusion was reached by Langer and Walker (1981) and their locations of the deletion endpoints are consistent with those described in section (b.ii). These authors constructed a restriction endonuclease cleavage map of pKM101, and their data for this part of the R46 molecule are consistent with those shown in Fig. 4.2. The sizes they reported for certain R46 restriction fragments not present in pKM101, however, differ markedly from those shown in Table 4.2. This is because the R46 isolate they examined (R46B) differs from that studied here (R46A) by inversion of a DNA segment of approximately 10 kb. All the restriction fragments whose size is altered by this inversion lie within the region of R46 deleted in pKM101 (Fig. 4.2, Fig. 4.5).

The DNA segment whose orientation differs in R46A and R46B is bounded by two copies of an 865 bp sequence in inverted orientation, referred to as ISN(a) and ISN(b) respectively. Homologous recombination across these two elements is probably responsible for inversion of the intervening region. Such inversions may potentially occur repeatedly, but are probably rare events since recA-dependent recombination across short regions of DNA homology is known to be inefficient. For example the

frequency of Hfr formation via recombination between IS elements on F and one of several homologous copies in the host chromosome is only approximately 10^{-6} per cell per generation (e.g. see Cullum and Broda, 1979). Moreover in restriction analysis of either R46A or R46B DNA, fragments specific to the alternative form of the plasmid are not visible on agarose gels (e.g. Fig. 4.4). The level of heterogeneity must therefore be less than 5-10%, but it is possible that some R46 DNA preparations contain both forms of the molecule. It might be possible to measure the relative proportions of R46A and R46B in such preparations by hybridisation of each form to restriction digests of R46 transferred to nitro-cellulose filters, but these experiments have not been attempted.

Regions of inverted homology on bacterial plasmids in many cases represent the terminal inverted repeats of transposable DNA segments and sometimes two complete copies of insertion sequence-like elements (see Ch. 7.a). Preliminary evidence is presented in Chapter 7 that ISN(b) on R46 is indeed an insertion sequence, and since ISN(a) is homologous it may have similar properties. One of the possible results of transposition events mediated by insertion sequences is the deletion of adjacent DNA segments. These deletions extend from one end of the transposable element for variable distances, such that the insertion sequence itself is not deleted (see Starlinger, 1980). One of the end-points in R46 DNA of the segment deleted in pKM101 lies at or extremely close to ISN(a). It is thus possible that the deletions that formed pKM101 were generated by ISN(a), and this element may therefore be an active insertion sequence as well as ISN(b). The presence on the N3 restriction cleavage map of two pairs of closely spaced SalI

and PstI sites (Fig. 4.6) raises the suggestion that this plasmid also may carry two copies of the ISN element. These possibilities are considered further in Chapter 7.

CHAPTER 5. CONSTRUCTION AND PROPERTIES OF PLASMIDS DERIVED FROM R46 AND N3

(a) Introduction

In order to construct combined physical and genetic maps of R46 and N3 showing the positions of their phenotypic determinants in relation to the restriction endonuclease cleavage sites, a variety of deletion, insertion and recombinant derivatives of the plasmids were generated. These contain defined regions of the parent molecules, determined by restriction cleavage analysis, and their phenotypes can be correlated with the presence or absence of these regions. This allows localisation of the phenotypic determinants on the R46 and N3 restriction maps. The construction and phenotypes of these various derivatives are described in this Chapter, and the resulting physical and genetic maps are presented in Chapter 6.

The plasmids derived from R46 and N3 fall into five classes: plasmids carrying deletions of specific restriction cleavage fragments, generated by in vitro recombination; plasmids with deletions extending from a single restriction cleavage site; recombinant clones of specific restriction fragments; plasmids carrying insertions of the transposon Tn5; and deletion plasmids generated in vivo.

(b) R46 derivatives: Construction and Phenotypes

(i) In vitro deletion plasmids

In vitro deletion derivatives of R46 were constructed using each of the restriction endonucleases HindIII, BamHI, EcoRI, SalI and BglII. Restriction cleavage fragments generated by one of

Table 5.1 : R46 in vitro deletion derivatives and rearrangements.

Plasmid	Restriction Fragment Composition ^a	Phenotype ^b								
		<u>Ap</u>	<u>Spc</u>	<u>Sul</u>	<u>Tc</u>	<u>Asa, Asi, Ant</u>	<u>Uvp</u>	<u>Ike, PR4^c</u>	<u>Tra</u>	<u>Sfx</u>
pED904	<u>HindIII</u> -A	R	R	S	S	R	+	S	+	
pED905	<u>HindIII</u> -A,B	R	R	S	R			S		
pED906	<u>HindIII</u> -A,C	R	R	R	S			S	+	
pED907	<u>BamHI</u> -A,B	R	R	R	S			S	+	
pED912	<u>EcoRI</u> -A,B	S	S	S	R	R	+	S	+	
pED918	<u>EcoRI</u> -A,D	R	R	S	S	S		S	+	
pED923	<u>SalI</u> -A	R	R	R	S	S		R	-	-
pED910	<u>BglIII</u> -D,E,G	R	R	S	S		-	R	-	
pED911	<u>BglIII</u> -B,E,G	S	S	S	R		-	R	-	
pED915	<u>BglIII</u> -D,E	R	R	S	S	S		R	-	
pED916	<u>BglIII</u> -B,E	S	S	S	R	S	-	R	-	
pED917	<u>BglIII</u> -B,C,D,E,G,I	R	R	S	R		-	R	-	
pED919	<u>BglIII</u> -A,B,C,D,E,F,G	R	R	S	R			S	± ^d	
pED920	<u>BglIII</u> -B,D,E,G,I	R	R	S	R			R	-	

a. See Fig.4.2 for map positions of restriction fragments.

b. R = resistant, S = sensitive. A blank space in the table indicates that the relevant test was not performed.

c. Sensitivity to the phages Ike and PR4 was determined by spot tests [Ch.2.(d)(i)]

d. pED919 was self-transmissible at approximately 1% of the frequency of R46: rearrangement of the BglIII fragments was responsible for this decrease.

these enzymes were ligated and used to transform ED395 or ED8654 with selection for Ap^{R} , Sp^{cR} , Sul^{R} or Tc^{R} . The plasmids recovered were expected to be composed of one or more fragments carrying the relevant antibiotic resistance gene, ligated to the fragment(s) containing the region of R46 essential for plasmid replication. Transformants selected on media containing one of the four antibiotics were replica-plated onto all four selective media to identify different plasmid phenotypes, and recombinant plasmids of different sizes were distinguished by their mobilities in agarose gel electrophoresis of small scale cleared-lysate preparations. Examples were then chosen for plasmid DNA purification. The restriction fragment composition of these deletion plasmids was established by comparing restriction digests of their DNA with equivalent digests of R46 DNA, using agarose gel electrophoresis (for example see Fig. 5.1). The resultant data is presented in Table 5.1, together with the plasmid phenotypes.

When HindIII and SalI were used in this procedure, with selection for Sp^{cR} transformants, plasmids composed of a single R46 restriction fragment were obtained (pED904 and pED923 are examples; see Table 5.1, Fig. 5.1).

The Sp^{cR} determinant and the replication region of R46 are therefore on the same HindIII and SalI fragments. In all other cases, the plasmids recovered contain two or more fragments. One fragment is common to all recombinant plasmids generated by a particular enzyme (e.g. see Fig. 5.1), and is thus inferred to include the replication region. The additional fragment(s) varied according to the resistance phenotype selected, and provided

Fig. 5.1 Restriction analysis of *in vitro* deletion derivatives of R46.

(a) Plasmids constructed using HindIII

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	λ 7	<u>HindIII</u>
2	pED906	<u>HindIII</u>
3	pED904	<u>HindIII</u>
4	pED905	<u>HindIII</u>
5	R46	<u>HindIII</u>

HindIII fragments A, B and C of R46 are indicated.

Sizes of HindIII fragments of λ 7 DNA are marked in kilobases.

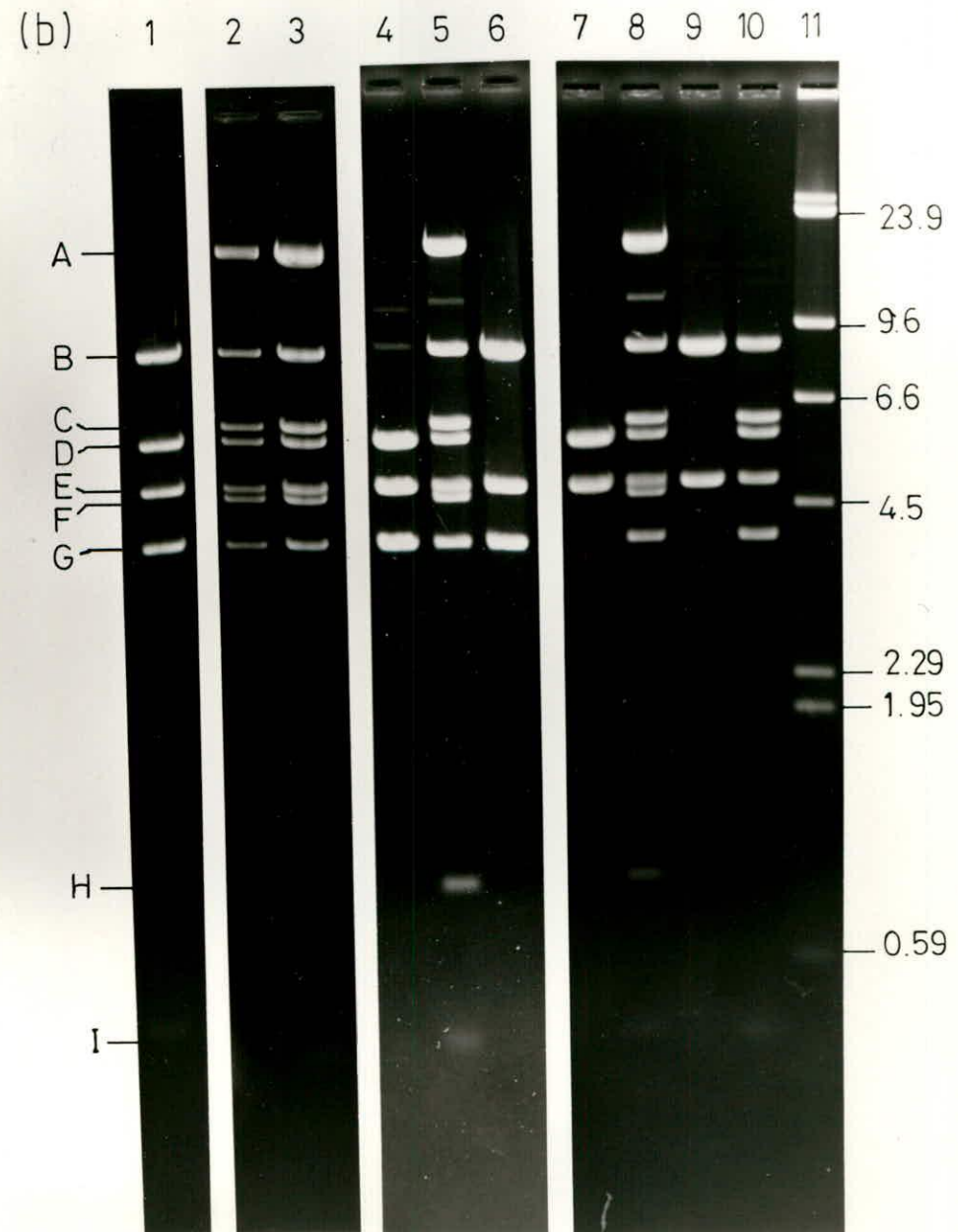
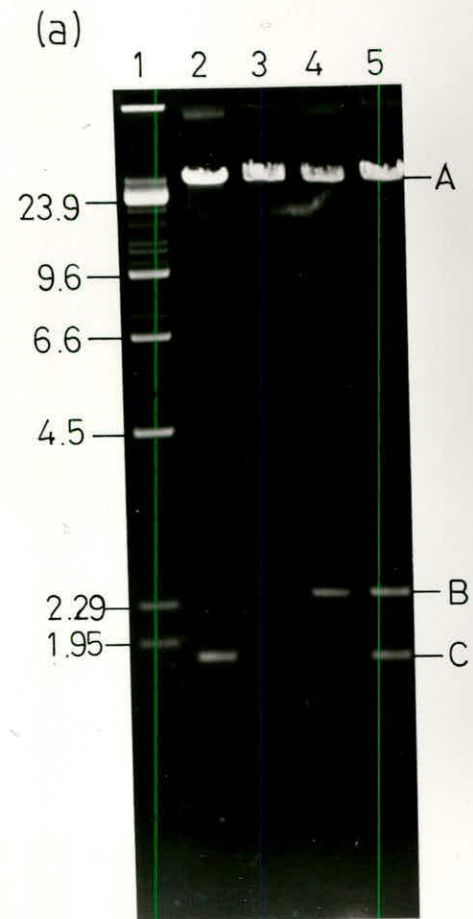
(b) Plasmids constructed using BglII

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pED920	<u>BglII</u>
2	pED919	<u>BglII</u>
3	R46	<u>BglII</u>
4	pED910	<u>BglII</u>
5	R46	<u>BglII</u>
6	pED911	<u>BglII</u>
7	pED915	<u>BglII</u>
8	R46	<u>BglII</u>
9	pED916	<u>BglII</u>
10	pED917	<u>BglII</u>
11	λ 7	<u>HindIII</u>

BglII fragments A to I of R46 are marked.

Note that all the plasmids shown contain BglII-E.

Fig. 5.1



preliminary information concerning the location of the relevant phenotypic determinants.

The order and orientation of restriction fragments in the above molecules was not in all cases the same as in R46, so that some of the plasmids represent rearrangements and multiple deletions of the parent R46 molecule. In addition certain plasmids, such as pED917 (Table 5.1), contained gratuitous restriction fragments unnecessary for the phenotype initially selected. Those deletion plasmids composed of two or more R46 restriction fragments that are contiguous in the parent molecule were further investigated to establish the relative orientation of these fragments. This was deduced from restriction cleavage analysis using enzymes having within the R46 fragment present in the deletion plasmid. recognition sites asymmetrically placed. / The fragments contained in pED904, 905 and 907 (Table 5.1) are in the same relative orientations as in R46, while the order of BglIII fragments in pED919 is different from that in R46. For example the KpnI-C fragment is not present in pED919, indicating that the fragments BglIII-A and BglIII-G are no longer contiguous in this plasmid (Table 5.1, Fig. 4.1). The relative orientation of EcoRI fragments A and D in pED918 (Table 5.1) could not be determined, because of the absence of asymmetrically placed restriction cleavage sites within EcoRI-D (Fig. 4.2).

(ii) Plasmids with deletions extending from a single restriction site

When linear plasmid DNA generated by a restriction endonuclease is used to transform E.coli cells, transformants are recovered at a very low frequency, and frequently contain plasmids that have suffered deletions of random length extending from one or both ends of the linear molecule (Thompson and Achtman, 1979).

Table 5.2 : Deletion derivatives of pED904.

<u>Plasmid</u>	<u>Size/kb</u>	<u>Phenotype^a</u>					
		<u>Ap</u>	<u>Spc</u>	<u>Asa, Asi, Ant</u>	<u>Uvp</u>	<u>Ike, PR4</u>	<u>Tra</u>
pED940	21.3	R	R	S	+	R	-
pED941	16.8	R	R			R	-
pED942	15.4	R	R				-
pED943	14.3	R	R		+		-
pED944	11.2	R	R				-
pED945	10.8	R	R		+		-
pED946	9.7	R	R		-		-
pED949	6.9	R	S		-		-
pED979	approx. 39.5	R		S			+
pED980	45.5	R		R			+
pED992	47.2	R					+
pED993	46.9	R					+

a. see footnotes to Table 5.1

This technique was used to generate a series of random deletions of pED904 from its single HindIII site. pED904 is an in vitro deletion plasmid composed of the HindIII-A fragment of R46 only; it determines resistance to arsenate ions, ampicillin and spectinomycin, and is self-transmissible (Table 5.1). In initial experiments the majority of plasmids recovered by this method did not carry deletions, but were identical to pED904, and control experiments showed that when unligated HindIII fragments of R46 DNA were used for transformation, molecules equivalent to pED904 with its single HindIII site were recovered. This indicated that linear molecules with HindIII cohesive ends could be transformed and precisely re-ligated in vivo. pED904 molecules made linear with HindIII were therefore treated with alkaline phosphatase to remove the 5'P termini and so prevent such ligation occurring. The DNA was then used for transformation of ED8654, and Ap^R and Spc^R transformants were each obtained at a frequency of about 10 per microgram of plasmid DNA.

The analysis of a series of deletion plasmids, pED940-pED949, obtained from one such experiment is described by Brown and Willetts (1981). These range in size from 6.9 kb (pED949) to 21.3 kb (pED940), and the segments of R46 DNA which they contain are shown in Fig. 6.1. Their phenotypes are listed in Table 5.2.

In a separate experiment a total of 32 Ap^R or Spc^R transformants was obtained from 4 µg of pED904. Replica-plate mating showed that 6 of these contained Tra⁺ plasmids, and four of these were further studied by restriction analysis: pED992 and pED993 carry small deletions of 0.2 kb and 0.5 kb, respectively, pED980 is deleted for 1.9 kb, and pED979 for approximately 8 kb (data

not shown). The 26 Tra^- transformants were screened genetically by spot tests (Ch. 2.c.iv) for the presence of oriT, the cis-acting sequence required for plasmid mobilisation in conjugation (see Chapters 1 and 8). These tests indicated that only three of the Tra^- plasmids were oriT⁺. In view of the location of oriT determined in Chapter 8, these Tra^- oriT⁺ plasmids probably carry deletions one end-point of which lies within the transfer region, and the Tra^- oriT⁻ plasmids must be deleted for the entire transfer region (see Fig. 6.6). A wide range of sizes of deletion can therefore be generated by this technique.

(iii) Recombinant clones of BglII restriction fragments from R46

BglII is the only restriction endonuclease tested that cleaves R46 at a fairly evenly distributed set of sites, the largest BglII fragment being 17.1 kb long (Table 4.2). Several of the nine BglII fragments were cloned in pBR325 (Ap^R Cm^R Tc^R). BglII fragments can be inserted into the BamHI site of this cloning vehicle since both BglII and BamHI produce 5'-terminal GATC single strand extensions; the insertion of DNA at this site eliminates the plasmid's Tc^R phenotype.

4 μg of R46 DNA digested with BglII was mixed with 1 μg of BamHI-digested pBR325 and ligated in a 100 μl volume. The mixture was used to transform ED8654 with selection for Cm^R , and transformants were screened for sensitivity to tetracycline. Approximately 20% of the Cm^R transformants were Tc^S . Small-scale/lysates were prepared from 37 such Cm^R Tc^S clones, and the approximate sizes of plasmid molecules determined from their mobilities in agarose gels. Representatives of each size class were then chosen for further study. The hybrid BamHI-BglII junctions between the

Table 5.3 : Recombinant clones containing restriction fragments of R46

<u>Plasmid</u>	<u>Composition</u>	<u>Phenotype^a</u>								
		<u>Ap</u>	<u>Cm</u>	<u>Spc</u>	<u>Sul</u>	<u>Tc</u>	<u>Km</u>	<u>Ike,PR4</u>	<u>Tra</u>	<u>Sfx</u>
pED926	pBR325 + <u>Bgl</u> III - H	R	R	S	S	S		R		
pED927	pBR325 + <u>Bgl</u> III - A	R	R	S	S	S		S	-	+
pED928	pBR325 + <u>Bgl</u> III - F	R	R	S	S	S		R	-	
pED929	pBR325 + <u>Bgl</u> III - D	R	R	R	S	S		R		
pED930	pBR325 + <u>Bgl</u> III - G	R	R	S		S			-	
pED933	pED931 ^b + <u>Kpn</u> I - C						R		-	
pED961	pBR322 + 2.85 kb <u>Eco</u> RI - <u>Sal</u> I fragment	R			R	S				
pED935	pBR325 + 23 kb <u>Pst</u> I fragment of pED953 ^c	S	R			R		S	+	+
pED936	pBR325 + 23 kb <u>Pst</u> I fragment of pED953 ^c	S	R			R		S	+	+

a. see footnotes to Table 5.1

b. see Chapter 2.k.i for construction of pED931

c. see Table 5.4 for pED953

vector DNA and the inserted fragment in these plasmids are not susceptible to cleavage by either enzyme, but there are sites for HindIII and SalI in pBR325 on either side of the cloned fragment. The sizes of HindIII + SalI double digestion fragments showed that BglII fragments, A, D, F, G and H had been cloned, and in some cases this was confirmed by the presence of specific additional restriction sites within the cloned fragment (data not shown). These recombinant plasmids are numbered pED926-pED930, and their properties are listed in Table 5.3. Fig. 5.3 shows a restriction map of pED927, a clone containing BglII fragment A; all the genes necessary for pilus synthesis are present on this fragment (Ch. 6.b.iv). A possible clone of the 0.35 kb BglII-I fragment was also identified, but none containing BglII-B, C or E. BglII-E contains the R46 replication region (see Chapter 6.b.i) and this might cause instability of a double replicon with pBR325 since a multicopy copy plasmid (pED981) carrying this region was unstable (K. Singh, personal communication). BglII-B includes the R46 Tc^R determinant (see Chapter 6.b.ii), and clones containing this fragment would not have been recovered since only Tc^S recombinant plasmids were chosen for analysis.

(iv) Tn5 insertion derivatives of R46, and in vivo deletion plasmids

A convenient method of obtaining transposon insertion derivatives of conjugative plasmids is to select appropriate transconjugants in conjugal crosses, using plasmid donor strains carrying a chromosomal transposon insertion. Transfer of a resistance marker on the transposon can occur as a result of its transposition onto the conjugative plasmid. Both Tra^+ and Tra^- plasmid insertion mutants can be recovered in this way, presumably

because the latter are complemented for transfer functions by other copies of the parent molecule in the donor cell (e.g. see Barth et al, 1978). In order to use this technique for the selection of R46 derivatives with insertions of the Km^R transposon Tn5, a chromosomal Tn5 strain, ED575, was first constructed, using a $\lambda::Tn5$ phage as the source of the transposon (see Ch. 2.j).

However, when R46 was introduced into ED575 by conjugation in two separate experiments, both the resulting strains were resistant to the IncN-specific phage Ike. These were subsequently shown to contain Tra^- deletion derivatives of R46, numbered pED973 and pED974, in which approximately 24 kb and 16.5 kb of R46 DNA had been deleted, respectively (Fig. 5.5). The deletion end-points lie within BglIII fragments A and B, and fragment A and G, respectively. Possible causes of these deletion events are considered in Chapter 7.

Since deletions of R46 were apparently generated at high frequency in ED575, R46::Tn5 plasmids were constructed by a two stage mating method in which the transfer of the Km^R marker from ED575 (R46) was selected immediately after the introduction of R46 into the strain. The Km^R marker should only be transmissible if it is inserted into R46 by a transposition event. J5 (R46) was first mated with ED575 for 30 mins. on a membrane filter (see Ch. 2.c.ii). The cells were then resuspended, mixed with the Nal^R final recipient strain ED3818 for a second 30 min filter mating, and Km^R [Nal^R] transconjugants selected. These were initially analysed by BglIII restriction digestion of plasmid DNA prepared from 1-5 ml cultures (Ch. 2.f.ii), and Fig. 5.2 shows examples of the agarose gel electrophoresis patterns obtained. All the R46::Tn5

Fig. 5.2

BglIII restriction analysis of plasmids carrying insertions of the transposon Tn5 (see Table 5.4). Electrophoresis was in 0.7% agarose gels.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pED958	<u>Bgl</u> III
2	pED959	<u>Bgl</u> III
3	R46	<u>Bgl</u> III
4	pED951	<u>Bgl</u> III
5	pED952	<u>Bgl</u> III
6	pED954	<u>Bgl</u> III
7	λ 7	<u>Hind</u> III
8	pED950	<u>Bgl</u> III
9	pED953	<u>Bgl</u> III
10	pED955	<u>Bgl</u> III
11	pED956	<u>Bgl</u> III
12	pED957	<u>Bgl</u> III
13	pED960	<u>Bgl</u> III
14	λ 7	<u>Hind</u> III

BglIII fragments A to G of R46 are marked, as is the 2.6 kb BglIII fragment from within the Tn5 DNA. The plasmid DNA used in these digestions was from unpurified small-scale preparations, hence RNA is visible towards the bottom of the gels. Sizes of HindIII fragments of λ 7 DNA are marked in kilobases.

Fig. 5.2

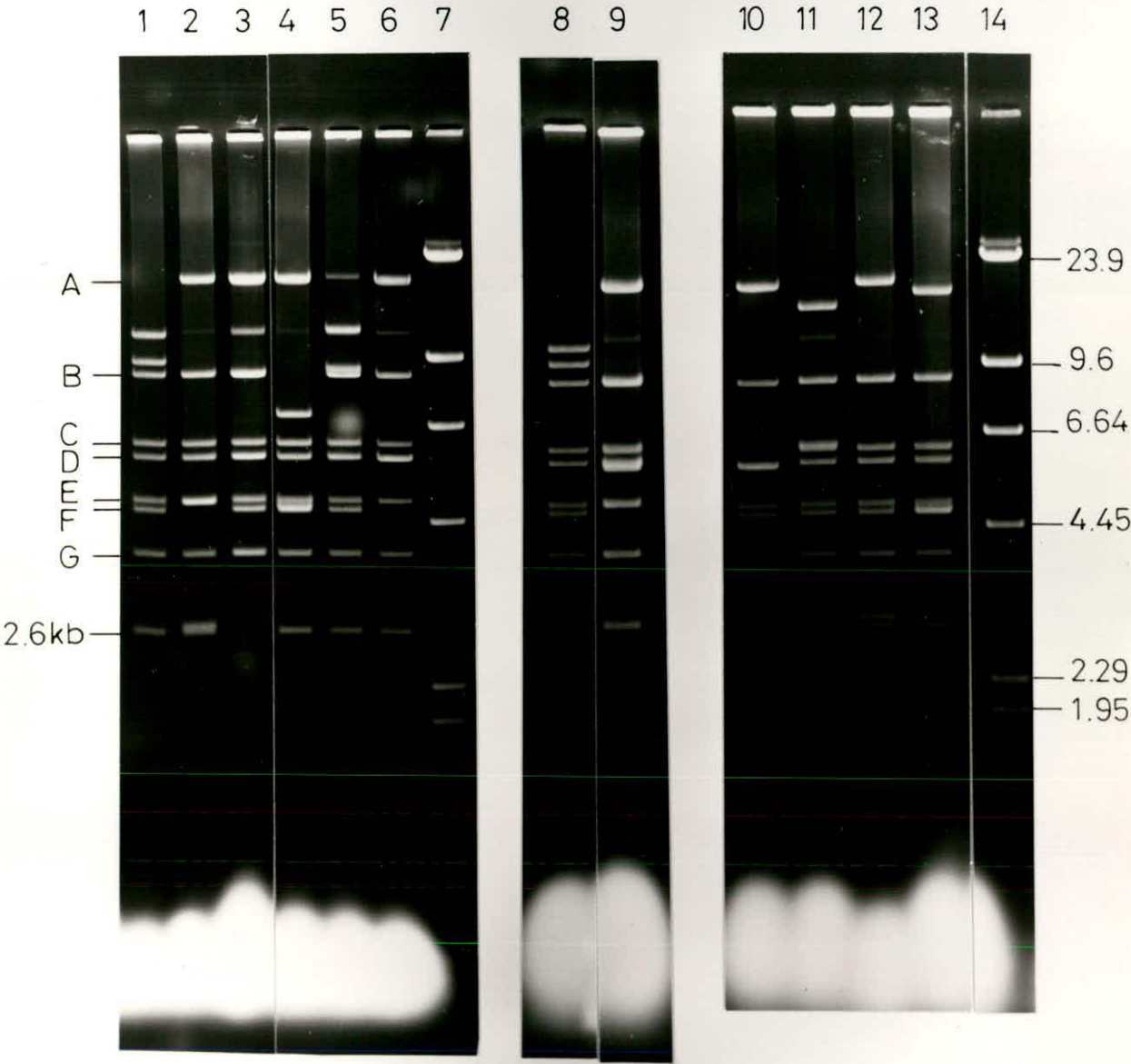


Table 5.4 : Insertions of transposon Tn5 in R46

<u>R46::Tn5 plasmid</u>	<u>Map co-ordinate of Tn5 insertion^b</u>	<u>Relevant Phenotype^a</u>		
		<u>Ike, PR4</u>	<u>Tra</u>	<u>Asa, Asi, Ant</u>
pED950	30.5	R	-	
pED951	41.7	S	+	R
pED952	31 .2	R	-	
pED953	13.45	S	+	
pED954	16.95	S	-	
pED955	8.85	S	+	
pED956	33.95			
pED957	37.15	S	+	R
pED958	29.5	R	-	
pED959	14.35	S	+	
pED960	35.35	S	+	

a. See footnote to Table 5.1. All R46::Tn5 plasmids listed are also Ap^R Spc^R Sul^R Tc^R

b. See Figs. 6.3, 6.6, 6.7.

plasmids contain a 2.6 kb BglIII fragment derived from the Tn5 DNA. In addition each plasmid contains all but one of the 9 BglIII fragments of R46, the missing fragment being replaced by two fragments of novel size caused by insertion of the transposon. The sizes of these novel fragments represent the distances from the BglIII sites in Tn5 to the flanking BglIII sites in R46. The data therefore locate each Tn5 insertion at one of two possible positions within a particular BglIII fragment of R46. Unambiguous map positions were determined from further restriction analysis using different enzymes (data not shown).

One advantage of the two-stage mating method used to generate these Tn5 insertions is that the probability of recovering two or more identical sibling R46::Tn5 plasmids in a single experiment is very low. A total of 26 Km^R[Nal^R] transconjugants were obtained in two experiments. 11 of the plasmids recovered were fully characterised, and each carries a Tn5 insertion at a different location. These plasmids are numbered pED950-pED960; their properties are shown in Table 5.4, along with the R46 map co-ordinates of their Tn5 insertions. Some of these are also indicated in Fig. 6.7.

Several attempts were made to obtain insertions of the Tp^R transposon Tn7 in R46 by isolating plasmids which transferred Tp^R from the strain J62::Tn7 (R46), but these were unsuccessful for reasons that are not clear.

(v) Recombinant clones containing the entire R46 transfer region

Tra⁺ recombinant plasmids containing the entire transfer region of R46 but not expressing IncN incompatibility are useful for two main reasons. Firstly they permit definition of the essential

transfer genes in R46. Secondly, they should co-exist stably with Inc^+ R46 derivatives and so facilitate genetic complementation studies of transfer-deficient mutations on the two plasmids.

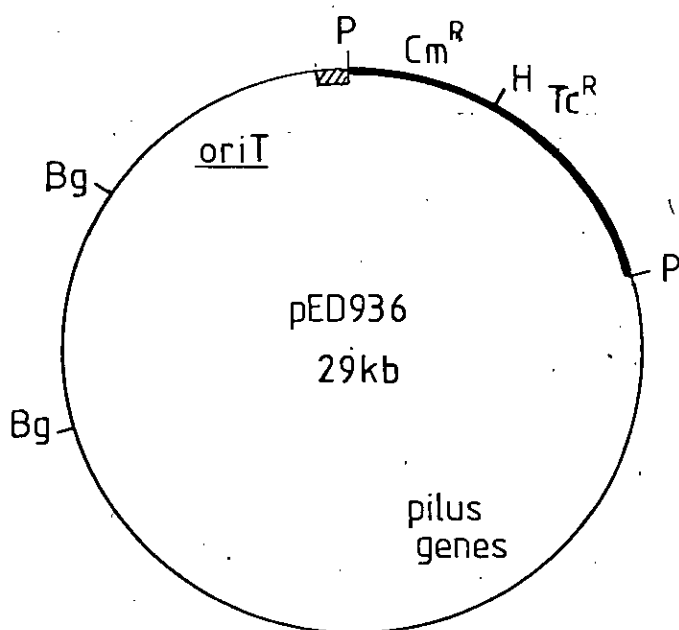
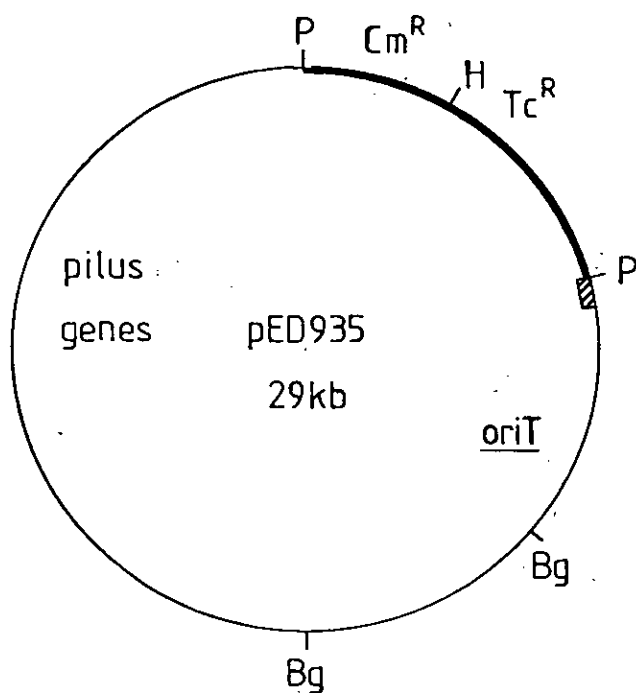
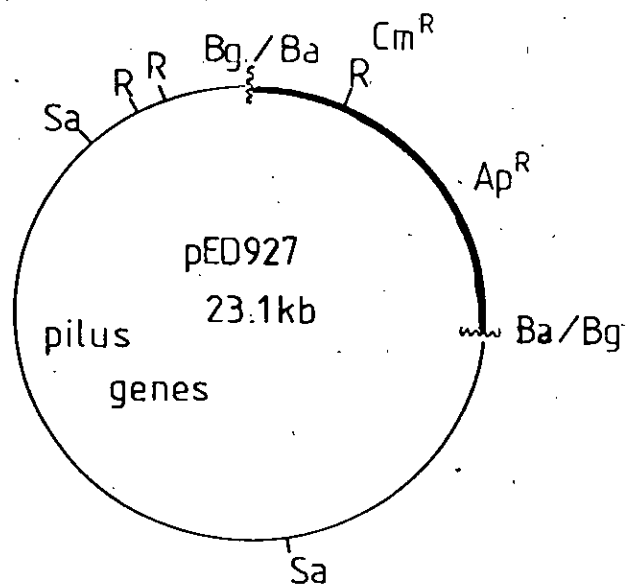
The phenotypes of deletion and recombinant derivatives of R46 allowed preliminary localisation of the R46 transfer genes between co-ordinates 0 and 36.6 kb (see Ch. 6.b.iv). Furthermore the transfer phenotypes of the R46::Tn5 plasmids described above, and the positions of their transposon insertions, suggested that if the transfer genes are clustered in a single region of R46 they must lie between the Tn5 insertion in pED953 and the PstI site at co-ordinate 35.95 kb (see Ch. 6.b.iv, and Fig. 6.6). The Tn5 DNA in pED953 contains a second conveniently placed PstI site, so PstI fragments of pED953 were cloned in the $\text{Ap}^R \text{Cm}^R \text{Tc}^R$ vector pBR325 on the assumption that all the genes required for plasmid transfer would be located on a single fragment. Self-transmissible Tc^R recombinant plasmids were selected by a procedure which is described by Brown and Willetts (1981): one such plasmid was pED935 (Table 5.3). This confers sensitivity to the phages I ϕ and PR4, and is self-transmissible at approximately 100% of the frequency of R46 (see below). Fig. 5.5 shows that pED935 contains a 23 kb PstI fragment from pED953 cloned in pBR325. pED935 is $\text{Cm}^R \text{Tc}^R$, but Ap^S since insertion of a DNA fragment at the PstI site of pBR325 inactivates the bla (β -lactamase) gene product of the vector. A second Tra^+ recombinant plasmid, pED936, was constructed, containing the 23 kb PstI fragment cloned in pBR325 in the opposite orientation to that in pED935. pED936 was derived by PstI digestion of pED935 DNA and re-ligation in vitro. Fig. 5.3 shows restriction maps of pED935 and pED936.

Fig. 5.3

Simplified maps of recombinant plasmids pED927, pED935 and pED936 sufficient to show the orientation of the cloned DNA in the pBR325 vector. pED927 contains BglIII fragment A of R46, and pED935 and 936 contain a 22 kb PstI fragment from pED953 (see Table 5.3).

pBR325 DNA is indicated by the thicker lines. The hybrid BamHI/BglIII junctions in pED927 are shown, but are not susceptible to cleavage by either enzyme. Cross-hatching indicates the approximately 0.5 kb of Tn5 DNA present in pED935 and pED936. The positions of the pilus genes and oriT are described in Chapters 6 and 8, respectively. Key to restriction enzyme sites: R, EcoRI; H, HindIII; Bg, BglIII; P, PstI; Sa, SalI; Ba, BamHI.

Fig. 5.3



Cells containing either pED935 or pED936 grow extremely slowly on minimal media, probably because these are multicopy plasmids and the effect of the Slo phenotype of R46 is accentuated by a gene dosage effect. The genetic determinant of this phenotype has recently been mapped within the segment of R46 cloned in pED935 (Langer et al, 1981). To overcome this slow growth the transfer frequencies of pED935 and pED936 were measured using nutrient selective agar for transconjugant selection, and donor cultures were grown in broth containing tetracycline to prevent plasmid loss, as some instability of pED935 was initially observed. No significant difference was apparent between the transfer frequencies of either of the two plasmids and that of R46 measured under the same conditions (data not shown). This suggests that pED935 and pED936 possess all the normal transfer functions of R46, and that these functions are expressed from their own promoter(s) rather than from the promoter of the pBR325 bla gene.

(vi) Multicopy Uvp⁺ recombinant plasmids

The Uvp determinant of R46 confers protection against the lethal effects of UV-irradiation and enhances the rate of UV and chemically induced mutagenesis (Ch. 3.b.v). The latter property has led to the incorporation of the R46 deletion derivative pKM101 in the Ames test for the detection of carcinogens as mutagens (McCann et al, 1975a; see Ch. 3).

Estimates of the amount of R46 DNA obtained from cleared lysate preparations, in relation to plasmids of known copy number, strongly suggest that the copy number of R46 is low, i.e. 1-5 copies per host chromosome (A. Brown and K. Singh, unpublished observations). Moreover Ando and Arai (1981) have recently reported the copy

number of the closely related Uvp^+ IncN plasmid N3 to be 2.8 per chromosome. The genetic determinant which mediates the Uvp phenotypes of R46 is therefore probably normally present in less than 5 copies per chromosome. It seemed possible that if the number of Uvp determinants in the cell were increased, the rate of chemically induced mutagenesis might be enhanced beyond the level determined by R46 or pKM101, allowing the development of a more sensitive bacterial test for the detection of carcinogens as mutagens. The Uvp determinant region of the R46 derivative pED945 (see section ii above, Table 5.2) was therefore cloned in the vector plasmid pBR327, which has a normal copy number of 20-40 per chromosome.

pED945 is the smallest of the deletion plasmids derived from pED904 (Table 5.1) that is Uvp^+ . It contains two EcoRI restriction fragments, one of which, approximately 9 kb in size, includes both the Uvp determinant and the R46 replication region (see Ch. 6, Fig. 6.1). This fragment was cloned into the EcoRI site of pBR327, and cells containing the relevant recombinant plasmids were selected as Tc^R transformants of the polA⁻ strain JG112 (see Fig. 5.4). R46 replicates normally in polA mutants (unpublished observations), while pBR327 replication is polA⁺-dependent (Kingsbury and Helinski, 1973). Consequently Tc^R plasmids in JG112 should be double replicons containing the Uvp^+ R46 Rep fragment ligated to the Tc^R pBR327 molecule.

These experiments were extended by K. Singh (Honours project, University of Edinburgh, 1981) and two plasmids, pED981 and pED982 were produced, corresponding to both orientations of the cloned EcoRI fragment. pED981 has a high copy number in the polA⁺ strain AB1157, but gives no greater protection against the lethal effects of

Fig. 5.4

Cloning strategy for construction of multicopy Uvp⁺ plasmids. The ~9 kb EcoRI fragment of pED945 containing the determinants for Uvp and Rep^R was cloned into the multicopy vector pBR327. Selection of Tc^R transformants in the polA strain JG112 ensured that the plasmids recovered would contain the Tc^R determinant of pBR327 linked to the polA-independent Rep region of pED945, and would therefore be Uvp⁺ double replicons. pED981 is one such example, and has a high copy number in the polA⁺ strain AB1157 (K. Singh and A. Brown, unpublished).

Key to restriction enzyme sites: R, EcoRI; Sa, SalI; Ac, AccI.

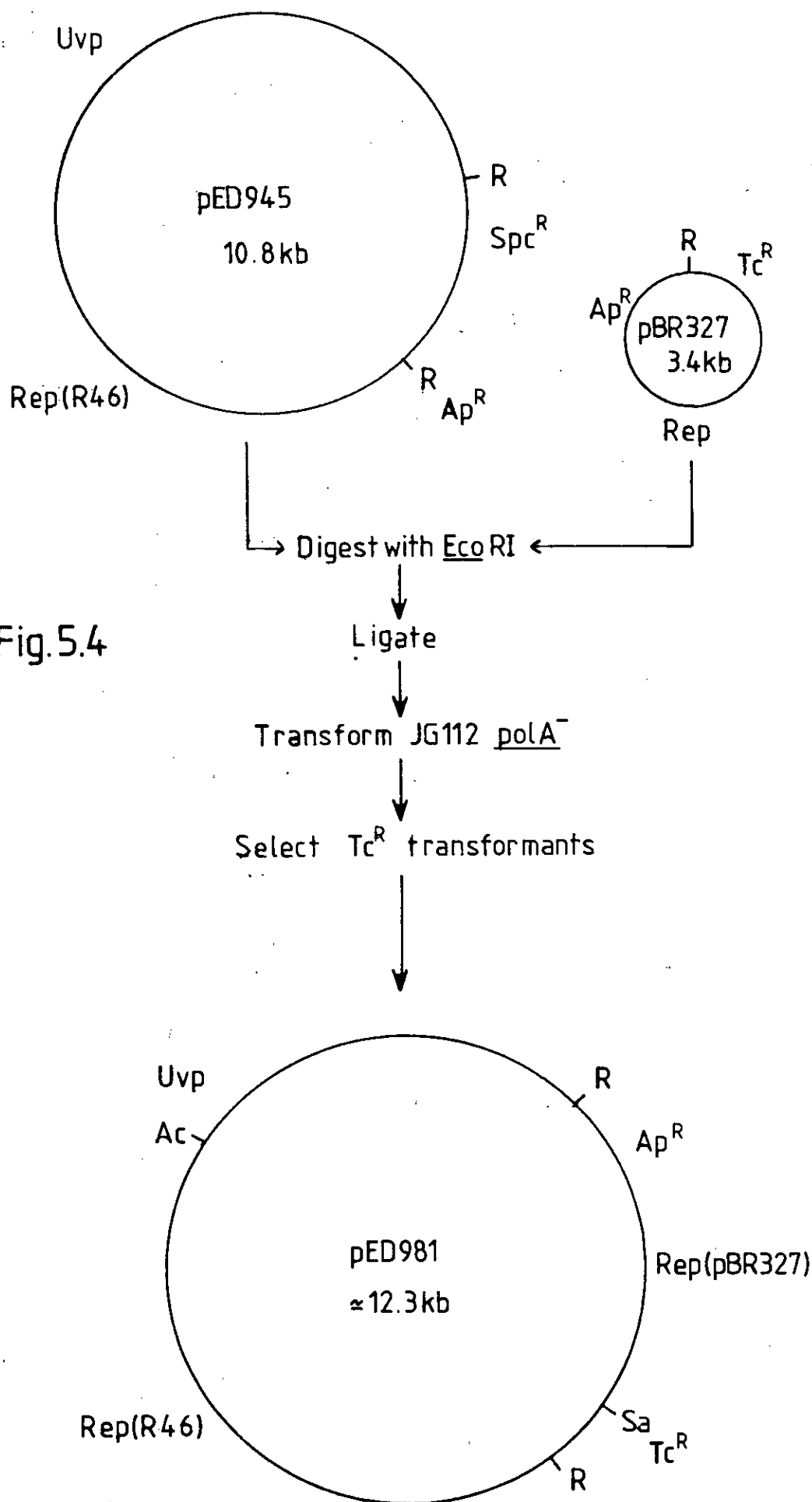


Fig.5.4

Fig. 5.5. 0.7% agarose gel electrophoresis

- (a) Restriction analysis of self-transmissible recombinant plasmids containing the 23 kb PstI fragment of pED953 cloned in pBR325.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pBR325	<u>Pst</u> I
2	pED935	<u>Pst</u> I
3	pED936	<u>Pst</u> I
4	pED953	<u>Pst</u> I
5	λ 7	<u>Hind</u> III

- (b) Restriction analysis of deletion derivatives of R46 generated in vivo.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pED973	<u>Bgl</u> II
2	R46	<u>Bgl</u> II
3	pED974	<u>Bgl</u> II

The sizes of R46 BglII fragments A to H are marked in kilobases.

Novel-sized fragments of 1.7 kb and 4.5 kb are visible in tracks 1 and 3, respectively.

- (c) Restriction analysis of spontaneous deletion derivatives of N3.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pED991	<u>Bgl</u> II
2	N3	<u>Bgl</u> II
3	pED990	<u>Bgl</u> II
4	λ 7	<u>Hind</u> III
5	pED990	<u>Pst</u> I
6	N3	<u>Pst</u> I
7	pED991	<u>Pst</u> I
8	λ 7	<u>Hind</u> III

N3 BglII fragments A to G are indicated next to track 1, and N3 PstI fragments A to F next to track 5.

The novel-sized BglII fragments in tracks 1 and 3 migrate close to or with BglII-B of N3.

Note that no novel-sized PstI fragment from pED991 is visible (track 7), suggesting that both deletion end-points lie close to PstI sites.

Sizes of HindIII fragments of λ 7 DNA are marked in kilobases.

Fig. 5.5

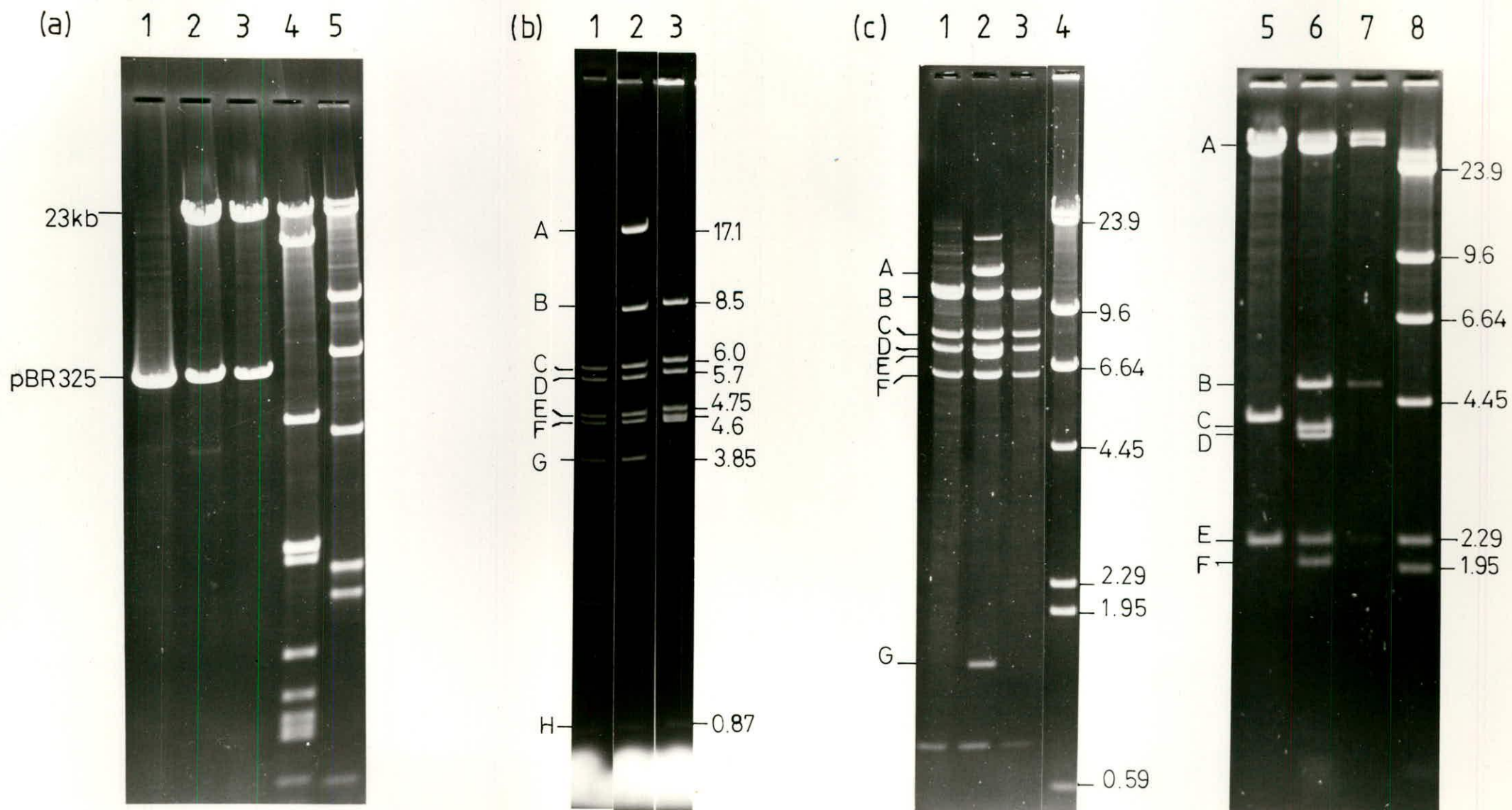


Table 5.5 : Plasmids derived from N3

<u>Plasmid</u>	<u>Composition/size</u>	<u>Phenotype^a</u>					
(a) <u>In vitro</u> deletion derivatives:		<u>Spc</u>	<u>Sul</u>	<u>Tc</u>	<u>Res (EcoRII)</u>	<u>Ike</u>	<u>Tra</u>
pED901	<u>Bgl</u> III - A,E	R	S	S	-	R	-
pED902	<u>Hind</u> III - A	S	S	R	-	S	+
pED903	<u>Hind</u> III - A,B,E	S	R	R	-	S	+
pED908	<u>Hind</u> III - A,B	S	R	R	-		
pED914	<u>Eco</u> RI - A	S	R	S	+	S	+
(b) Deletion derivatives of pED902:							
pED971	approx.33kb			R		R	-
pED972	18.3kb			R			-
(c) Spontaneous deletion derivatives:							
pED990	approx.47kb	R		S			
pED991	approx.46kb	R	R	S		S	

a. See footnote to Table 5.1.

UV-irradiation than does pED945. However, cells containing pED981 show a ten-fold increase in spontaneous and MMS-induced mutagenesis relative to cells containing pED945 (K. Singh and A. Brown, unpublished). The implications of these results are discussed in Chapter 6.d.

(c) N3 Derivatives: Construction and Phenotypes

(i) In vitro deletion plasmids

Deletion derivatives of N3 were constructed in vitro by methods similar to those employed for R46 (see Ch. 5.b.i), using each of the restriction enzymes HindIII, EcoRI, and BglII, with selection for Spc^R , Tc^R or Sul^R recombinants. The restriction fragment composition and phenotypes of these plasmids are shown in Table. 5.5

(ii) Plasmids with deletions from a single restriction site

pED902 is a Tc^R in vitro deletion plasmid composed of the HindIII-A fragment of N3 only (Table 5.5). A series of deletions were generated from the single HindIII site in this plasmid, by transformation of ED8654 with pED902 DNA made linear with HindIII and treated with alkaline phosphatase as described for pED904 in section b.ii above. A total of 23 Tc^R transformants were obtained, of which 10 contained plasmids smaller than pED902 as determined from agarose gel electrophoresis of cleared lysate preparations. These 10 plasmids were all Tra^- . The remaining transformants contained Tra^+ plasmids indistinguishable in size from pED902 on gels but may nevertheless have carried small deletions. The smallest and largest Tra^- plasmids were numbered pED972 and pED971 respectively, and analysed by restriction digestion. The remaining 8 were stored. pED972 is 18.3 kb and pED971 approximately 34 kb

in size; the regions of N3 included in these plasmids are indicated in Fig. 6.10.

(iii) Spontaneous deletion derivatives of N3

During the course of this study two spontaneous Tc^S derivatives of N3 were detected fortuitously, both of which are deletion mutants. The first, pED990, was identified during routine strain testing of single colonies of ED395 (N3). The second was recognised during contour length measurements of N3 molecules in the electron microscope. 1 in 12 of the molecules measured in one such experiment was at least 10 kb smaller than the mean length of N3 (these were ignored in calculations of this mean). Single colonies of the strain from which this DNA had been prepared were tested by replica-plating, and 1 out of 100 was Tc^S . The plasmid in this colony was numbered pED991. Both pED990 and pED991 contain only a single EcoRI cleavage site, and BglIII restriction analysis shows they have suffered deletions of approximately 12 kb and 11 kb respectively (Fig. 5.5). The PstI restriction cleavage patterns shown in Fig. 5.5 suggest that the end-points of the deletion suffered by pED990 lie within PstI fragments C and B, and that those of the pED991 deletion lie close to each of PstI sites at co-ordinates 51.75 and 5.4 kb (Fig. 4.6). The possible significance of these data is discussed in Chapter 7.

(d) Discussion

As a consequence of the uneven distribution of restriction sites in R46 and N3 and the map positions of their replication regions (Figs. 6.1, 6.9) the in vitro deletion plasmids produced using EcoRI and HindIII carried relatively small deletions in the same region of each parent plasmid (Tables 5.1, 5.6; Figs. 4.2, 4.6).

In order to obtain deletions with end-points within the segments of R46 and N3 containing relatively few restriction sites, E.coli cells were transformed with linear plasmid DNA. This generated deletions from one or both ends of the linear molecules. The mechanisms by which deletion and recircularisation of the DNA occurs in vivo are not known. When starting with a 47.4 kb plasmid (pED904), the length of DNA deleted varied from 0.2 kb (pED992) to 40.5 kb (pED949), so this is a useful technique for constructing deletions of a wide range of sizes. The minimum size of the deletion plasmids recovered was limited by the distance between the replication region of the parent molecule and the genetic determinant of the phenotype selected.

The well-spaced series of BglII sites in R46 provided suitably sized restriction fragments for cloning, and recombinant plasmids were constructed containing each of at least 5 different R46 BglII fragments in pBR325. The scarcity of other restriction sites in the region of R46 containing the conjugal transfer genes (ch. 6.b.iv) raised the possibility of cloning the entire transfer region on a single restriction fragment. The smallest R46 fragment containing these genes, however, is the very large PstI-A fragment (36.5 kb). The transposon Tn5 was therefore used to provide a conveniently placed restriction site, and the transfer region was cloned on a single 23 kb PstI fragment from the R46::Tn5 plasmid pED953. This produced self-transmissible multicopy recombinant plasmids (pED935 and pED936).

CHAPTER 6. PHYSICAL AND GENETIC MAPS OF R46 and N3

(a) Introduction

A number of plasmids derived from R46 and N3 by deletion, insertion, or in vitro recombination were described in Chapter 5, and the regions of the parent molecules contained in them were defined by restriction cleavage analysis in conjunction with the restriction maps of R46 and N3 presented in Chapter 4. The phenotypes determined by these various derivatives can be attributed to the regions of R46 or N3 DNA they contain, and equally the lack of certain phenotypes of the parental plasmids can be related to the loss or insertional mutation of particular DNA segments. In this manner the genetic determinants of plasmid phenotypes and functions were located on the R46 and N3 restriction maps, and combined physical and genetic maps of the two plasmids were constructed.

(b) R46: Locations of Genetic Determinants on the Physical Map(i) The replication region

The segment of R46 common to all the deletion derivatives constructed was assumed to contain the DNA essential for plasmid maintenance and replication, and presumably includes a replication origin. This segment is approximately 3.4 kb long, within BglIII fragment E, from co-ordinate 2.2 kb to approximately 5.6 kb (Fig. 6.1, and see Fig. 5.1). The detailed reasons for this conclusion are described by Brown and Willetts (1981).

The smallest R46 derivative constructed was pED949, which constitutes a 6.9 kb mini-R46 replicon. pED949 expresses IncN incompatibility and is stably maintained in non-selective media over

Fig. 6.1

Expanded map of R46 from co-ordinates 49 to 20 kb showing the lengths of R46 DNA contained in deletion plasmids derived from pED904, the location of the replication region (Rep), and the positions of the genetic determinants of ampicillin resistance, spectinomycin resistance, and UV-protection. The R46 DNA contained in the multicopy Uvp⁺ plasmid pED985 is also shown, as are the positions of the short inverted repeats at approximately co-ordinates 2.4 and 10.1 kb. From the available data it was not possible to establish whether or not the left-hand repeat lies within the essential replication region.

Dotted lines indicate uncertainties in the precise end-points of deletion plasmids and phenotypic determinants. Restriction fragments relevant to the text are marked; arrowheads indicate that a fragment extends off the figure. Co-ordinates are marked in kilobases. For key to restriction enzyme sites see legend to Fig. 6.6, and Ac = AccI.

pED940 ---
 pED941 ---
 pED942 ---
 pED943 ---
 pED944 ---
 pED945 ---
 pED946 ---
 pED949 ---

Fig. 6.1

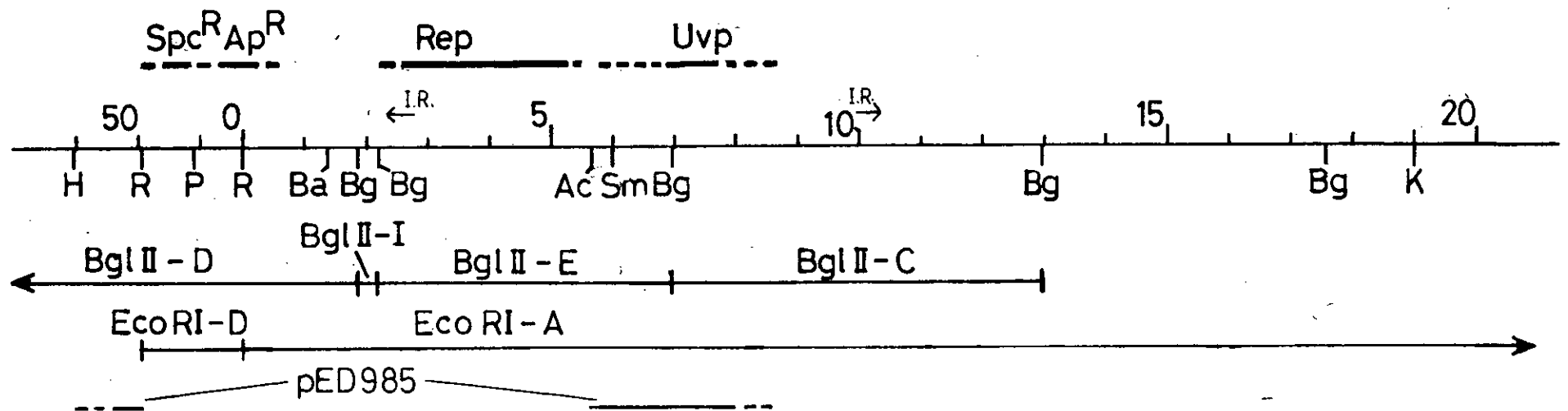


Fig. 6.2

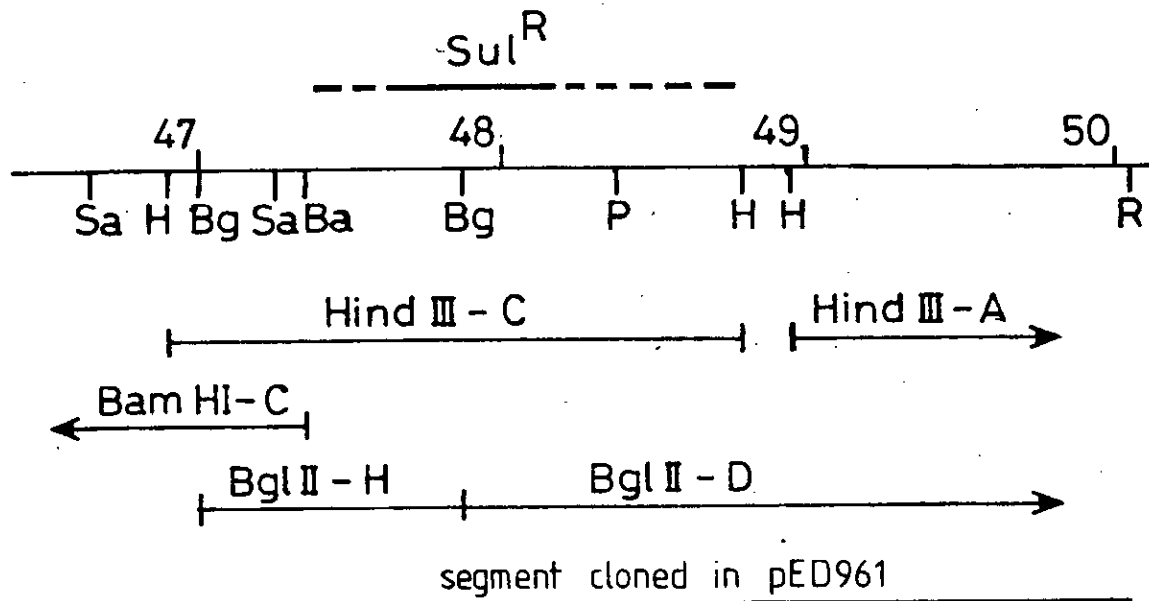


Fig. 6.2

Expanded map of the sulphonamide resistance region of R46, showing the position of the Sul^R determinant, named restriction fragments relevant to the text, and the cloned DNA segment contained in pED961. Dotted lines indicate uncertainties in the precise end-points of the Sul^R determinant. Arrowheads indicate that a restriction fragment extends off the figure.

Co-ordinates are marked in kilobases. For key to restriction enzyme sites see legend to Fig. 6.4.

approximately 20 generations. Longer term stability experiments were not performed with R46 or any of its derivatives, so it is not known whether or not these plasmids carry a par function (see Chapter 1.).

(ii) Antibiotic resistance determinants

Ampicillin resistance and spectinomycin resistance

The map positions of these determinants on R46 are described by Brown and Willetts (1981). The Spc^R determinant is located entirely within EcoRI fragment D, while the determinant of Ap^R lies at least partly within this fragment and may extend into EcoRI-A, as shown in Fig. 6.1. Langer *et al* (1981) have reported that the Ap^R determinant of pKM101 includes the EcoRI site equivalent to that between EcoRI-A and EcoRI-D of R46. This is consistent with the data above.

Sulphonamide resistance

The restriction fragment composition and phenotypes of the in vitro deletion plasmids pED904, 906, 907, 915 and the recombinant clones pED926 and pED929 (Tables 5.1, 5.3), allowed the entire Sul^R determinant to be mapped within a 1.4 kb segment of HindIII fragment C of R46 (Brown and Willetts, 1981; see Fig. 6.2). This segment is bounded by BamHI and HindIII sites, and contains single cleavage sites for BglII and PstI. This BglII site lies within BglII fragments H and D (see Fig. 6.2) and plasmids containing either one of these fragments (e.g. pED926, and pED929, respectively; Table 5.3) are not Sul^R , indicating that the BglII site lies within the Sul^R determinant.

An $\text{Ap}^R \text{ Sul}^R$ recombinant plasmid, pED961, was constructed by cloning a 2.8 kb EcoRI-SalI double digestion fragment of R46 in

the vector plasmid pBR322 (see Ch. 2.k.ii). This fragment includes the 1.4 kb Sul^R region (Fig. 6.2), and the only BglIII site in pED961 is that within the Sul^R determinant. Insertion of BglIII restriction fragments at this site in pED961 inactivates Sul^R (P. Meulien, personal communication). This confirms that the BglIII site lies within the determinant, and also demonstrates the value of pED961 as a BglIII cloning vehicle since insertional inactivation of Sul^R allows the ready detection of recombinant clones.

Tetracycline resistance

The Tc^R plasmid pED912 is composed of R46 fragments EcoRI-A and EcoRI-B (Table 5.1) which are not contiguous in the parent R46 molecule (Fig. 4.2). pED918 is composed of EcoRI-A and EcoRI-D and is Tc^S (Table 5.1). The Tc^R determinant of pED912 is thus located within EcoRI-B. Tc^R is not expressed from the HindIII-A fragment in pED904, although this contains all of EcoRI-B except the small terminal HindIII-EcoRI segment, measured as approximately 30 base pairs on polyacrylamide gels (Table 5.1, Fig. 4.1). This fixes one end of the Tc^R determinant between the HindIII site and adjacent EcoRI site at the extreme right hand end of EcoRI-B (Fig. 6.3). As expected, pED905, consisting of the fragments HindIII-A and HindIII-B ligated in their original orientation, does confer Tc^R (Table 5.1).

The restriction map of this region of R46 is indistinguishable from that of the Tc^R gene(s) of the plasmid pSC101 (Tait and Boyer, 1978) as shown by the co-migration of the EcoRI-HindIII, HindIII-BamHI, and HindIII-SalI double digest fragments in gel electrophoresis (Fig. 6.4). Assuming that the Tc^R determinants are of similar size, the left hand end of the R46 Tc^R determinant is located at approximately co-ordinate 43.1 kb (Fig. 6.3). The data shown in Figs. 6.3 and 6.4 strongly suggest that the Tc^R

resistance determinant of R46 is of the Class-C variety defined by Mendez *et al* (1980), and may be virtually identical to that of pSC101. The promoter of the Tc^R gene(s) of pSC101 lies within the 31 bp HindIII-EcoRI double digestion fragment shown in Fig. 6.4 (Sutcliffe, 1979); the location of one end of the R46 Tc^R determinant in an equivalent fragment of the same size indicates that the two determinants may have similar structure.

Resistance to Arsenate, Arsenite and Antimony (III)
deletion

The R46 in vitro/derivatives pED912 and pED918 are composed of EcoRI fragments A + B / A + D respectively (Table 5.1). pED912 determines resistance to arsenate, arsenite and antimony (III) salts, while cells carrying pED918 are sensitive to all three compounds (Table 5.1). Since EcoRI-A and EcoRI-B are not contiguous in the R46 molecule (Fig. 4.2), these data indicate that the genetic determinants for Asa^R , Asi^R and Ant^R all lie within EcoRI-B. This fragment contains a single site for the enzyme BglIII (Fig. 4.2) and each of the BglIII fragments bounded by this site (BglIII-A and BglIII-B) were therefore tested for their ability to confer Asa^R , Asi^R or Ant^R , in the plasmids pED927 and pED916. pED927 is a recombinant clone of BglIII-A in pBR325 (Table 5.3) and pED916 is composed of the BglIII-B and BglIII-E fragments only (Table 5.1). Although these plasmids sometimes showed variable intermediate levels of resistance, neither of them conferred definite resistance to arsenate, arsenite or antimony (III), so continuity of DNA across the BglIII site between BglIII-A and BglIII-B is important for full expression of all three metal ion resistances.

The properties of the deletion plasmids pED980 and pED979 (Table 5.2) corroborated the map position of the three determinants.

pED980 contains most of EcoRI-B, including the BglIII site between BglIII-A and BglIII-B (Fig. 6.3), and is $\text{Asa}^R \text{Asi}^R \text{Ant}^R$. pED979 is deleted for the relevant BglIII site and for most of EcoRI-B (Fig. 6.3), and is $\text{Asa}^S \text{Asi}^S \text{Ant}^S$ (Table 5.2). The R46::Tn5 plasmids pED951 and pED957 (Table 5.4), which each contain Tn5 insertions within EcoRI-B, both confer resistance to Asa, Asi and Ant; the three resistance determinants probably lie between these two insertions (Fig. 6.3).

None of the deletion and insertion derivatives of R46 so far tested have separated the Asa^R , Asi^R and Ant^R determinants genetically, and a possible interpretation of the data is that resistance to the three compounds is determined by two or more genes organised in an operon. Previous studies of plasmid-borne resistance to Asa, Asi and Ant have mostly been conducted with a group of plasmids in Staphylococcus aureus. The resistances to arsenate and arsenite in these plasmids are genetically separable (Löfdahl et al, 1978; Silver et al, 1981), and Novick et al (1979a, 1979b) have reported evidence that the genes for $\text{Asa}^R \text{Asi}^R$ and Ant^R are organised in an operon of two or more genes.

In E.coli the $\text{Asa}^R \text{Asi}^R$ and Ant^R phenotypes determined by plasmids R773 and R828 show strikingly general similar/characteristics to those determined by plasmids in S.aureus (Silver et al, 1981). Arsenite resistance in E.coli is probably mediated by a different mechanism from that determining resistance to arsenate (Silver et al, op.cit.), and the Asa, Asi and Ant resistance determinants of E.coli plasmids may also be organised in an operon. The data presented above for R46, however, is insufficient to establish this.

Fig. 6.3

Expanded map of R46 between co-ordinates 36 and 45 kb showing the position of the tetracycline resistance determinant, and the approximate location of the arsenate, arsenite and antimony(III) resistance determinant(s). The relevant R46 DNA contained in the deletion plasmids pED979 and pED980 is marked, as are named restriction cleavage fragments relevant to the text. The triangles mark the position of the Tn5 insertions in pED957 and pED951.

Arrowheads indicate that a plasmid or restriction fragment extends off the figure. For key to restriction sites see legend to Fig. 6.6. The positions of the five PstI sites between co-ordinates 38.7 and 41.3 kb were not determined.

Fig. 6.3

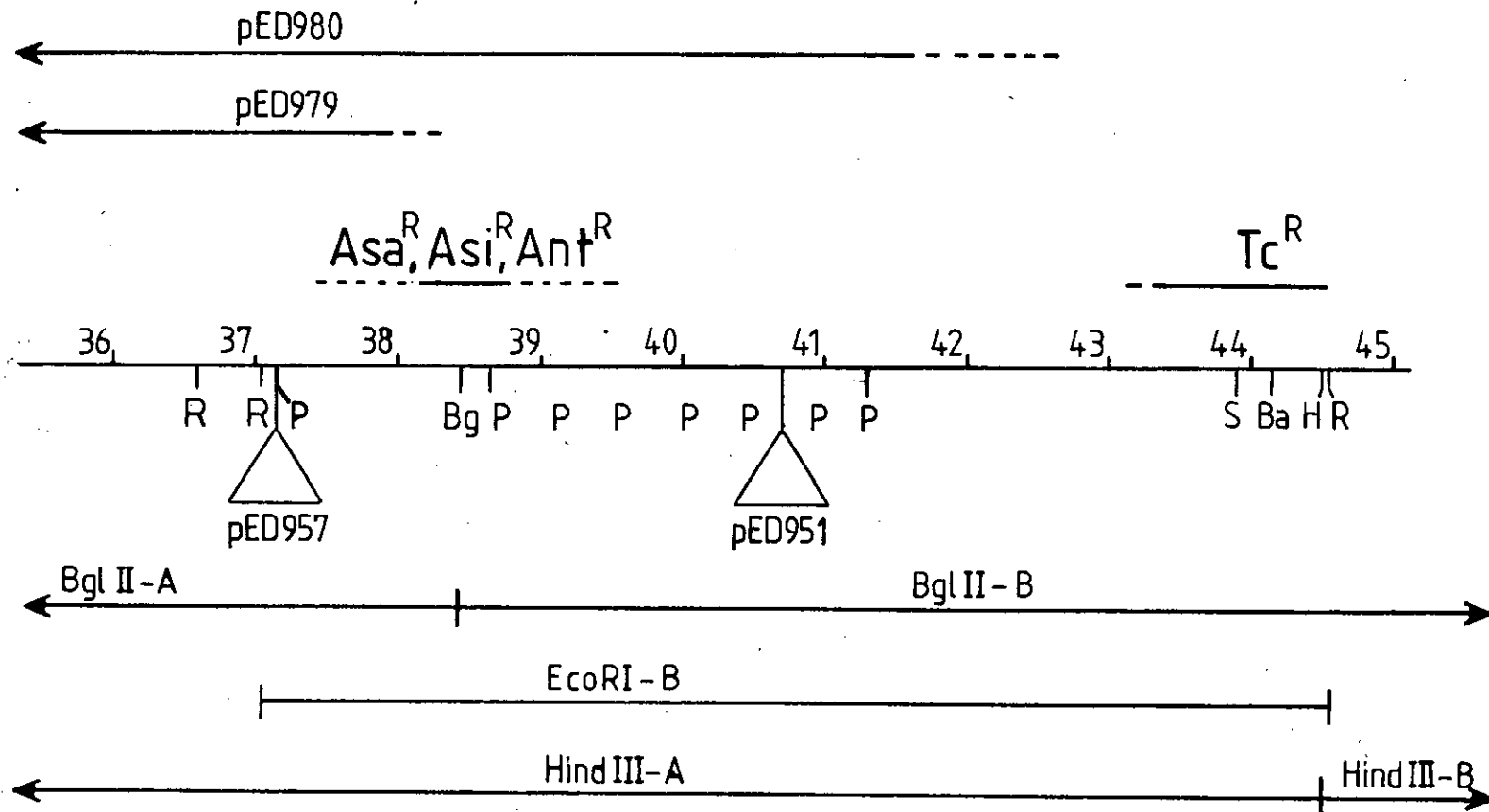


Fig. 6.4. Comparative restriction analysis of the Tc^R regions of R46 and pSC101.

- (a) 0.7% agarose gel electrophoresis of HindIII + SalI double digestion products

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>
1	λ 7	<u>HindIII</u>
2	pBR322	<u>HindIII</u> + <u>SalI</u>
3	pED912	<u>HindIII</u> + <u>SalI</u>
4	pSC101	<u>HindIII</u> + <u>SalI</u>
5	pED912	<u>HindIII</u> + <u>SalI</u>

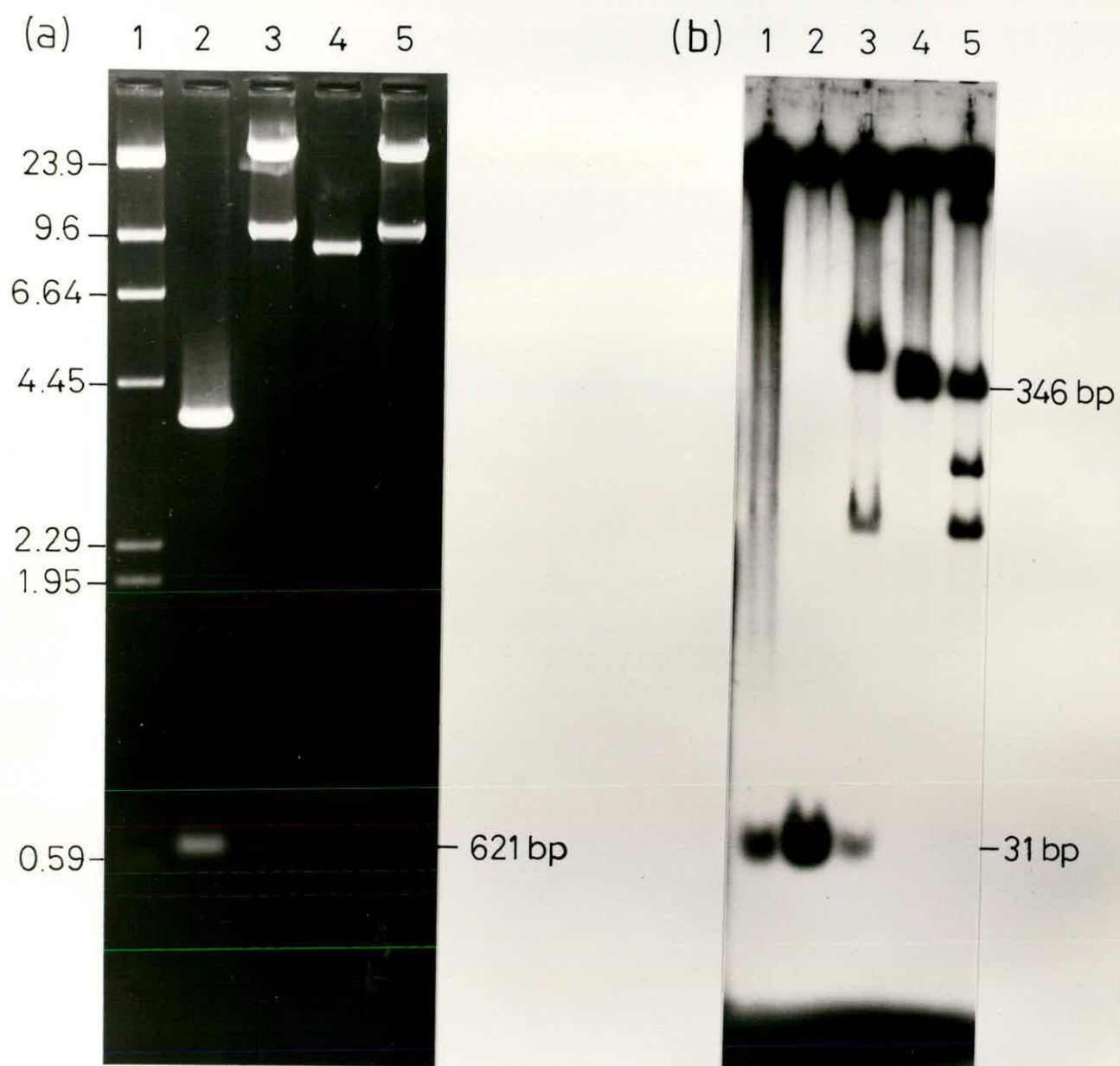
Note that the Tc^R determinants of pBR322 and pSC101 are identical, and the 621 bp fragment marked is derived from these determinants (Sutcliffe, 1978, 1979). A fragment of identical size is also present in pED912.

- (b) Autoradiogram of [³²P]-end-labelled restriction fragments after electrophoresis in an 8% polyacrylamide gel.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonucleases</u>
1	pSC101	<u>EcoRI</u> + <u>HindIII</u>
2	pBR322	<u>EcoRI</u> + <u>HindIII</u>
3	R46	<u>EcoRI</u> + <u>HindIII</u>
4	pBR322	<u>HindIII</u> + <u>BamHI</u>
5	R46	<u>HindIII</u> + <u>BamHI</u>

Note that the 346 bp and 31 bp fragments from within the Tc^R region of pBR322 comigrate with fragments derived from the equivalent region of R46.

Fig. 6.4



(iii) The Uvp determinant

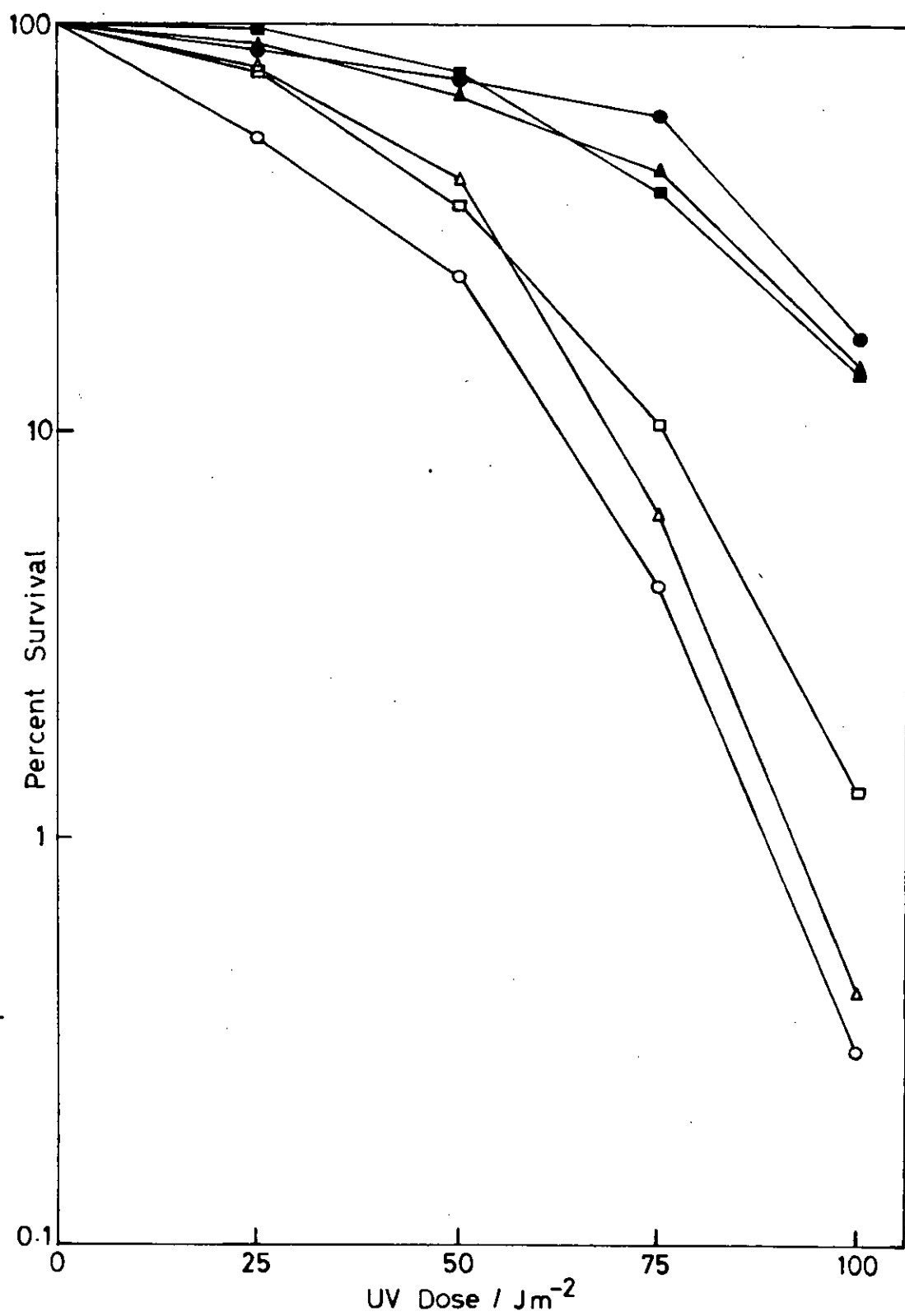
All the Uvp phenotypes of R46 are probably mediated by a single plasmid gene product (see Ch. 3.b.v), so R46 derivatives conferring UV-protection are assumed below to determine all the other Uvp phenotypes as well. The location of the Uvp determinant was deduced by testing the ability of recombinant and deletion derivatives of R46 to protect AB1157 from the lethal effects of UV-irradiation. On the basis of UV dosage/survival curves (Fig. 6.5) the plasmids clearly fell into two classes, judged to be Uvp^+ and Uvp^- with reference to AB1157 (R46) and AB1157 controls. These data, in conjunction with restriction analysis of the plasmids, allowed the Uvp determinant to be mapped. The reasoning involved is described by Brown and Willetts (1981). The region of R46 DNA essential for the Uvp phenotype is located at least in part between co-ordinates 6.8 and 8.7 kb (Fig. 6.1), and includes the BglII site at 6.95 kb (Fig. 6.1).

The smallest of the Uvp^+ R46 deletion plasmids constructed was pED945 (Fig. 6.1), and the Uvp determinant and replication region was cloned from this plasmid into pBR327 to form pED981 (A. Brown and K. Singh, unpublished, see Ch. 5.b.vi). K. Singh (Honours project, University of Edinburgh, 1981) subsequently constructed a deletion derivative of pED981, numbered pED985, which is also Uvp^+ , and from which the pED945 DNA from the EcoRI site at 50.05 kb to the AccI site at 5.6 kb (Fig. 6.1) is deleted. Since other Uvp^+ R46 derivatives (e.g. pED912) lack the small segment of pED945 DNA located anticlockwise of R46 co-ordinate 50.05 kb (see Fig. 6.1) these data locate all the R46 DNA sufficient for a Uvp^+ phenotype within the 3.1 kb segment between co-ordinates 5.6 and 8.7 kb (Fig. 6.1).

Fig. 6.5: Ultraviolet dosage-survival curves of AB1157 strains containing R46 derivatives ●, R46; ■, pED912; ▲, pED945; ○, no plasmid; Δ, pED946; □, pED917 (see Tables 5.1, 5.2).

pED912 and pED945 were judged to be Uvp⁺, and pED946 and pED917 Uvp⁻. These data allowed the Uvp determinant to be localised as shown in Fig. 6.1 (see Brown and Willetts, 1981).

Fig. 6.5



Shanabruch and Walker (1980) have located a region necessary for Uvp phenotypes within a 2.2 kb segment of pKM101, which corresponds to that between co-ordinates 5.65 and 7.85 kb on R46. This is consistent with the data presented here. Also Langer et al (1981) have recently defined a region of pKM101 sufficient to produce the Uvp phenotypes; this is equivalent to the R46 DNA from co-ordinates 2.0 to 7.8 kb, and includes the 3.1 kb segment demonstrated above to be sufficient for a Uvp⁺ phenotype.

(iv) Conjugal transfer genes

The R46 deletion plasmids pED912 and pED918 are both Tra⁺ and have only the EcoRI-A fragment in common (Table 5.1). Consequently all the genes required for R46 transfer are most probably located between co-ordinates 0 and 36.6 kb (Fig. 6.6). The deletion plasmid pED923, composed of the SalI-A fragment, is Tra⁻ (Table 5.1), implying that at least some of the transfer genes lie clockwise of the SalI site at co-ordinate 25.7 kb (see Fig. 6.6). There must also be a region of DNA essential for transfer anticlockwise of the BglII site at co-ordinate 21.4 kb (see Fig. 6.6) since pED927, a recombinant clone containing BglII-A, is also non-transmissible (Table 5.3).

These data, together with the phenotypes and map positions of the Tn₅ insertions described in Chapter 5.b.iv, suggested that the essential transfer genes could lie in a single region of R46 between the position of the Tn₅ insertion in the Tra⁺ R46::Tn₅ plasmid pED953 and the PstI site at co-ordinate 35.95 kb (Figs. 6.6, 6.7). This assumption was confirmed by the construction of pED935, a self-transmissible recombinant clone (see Ch. 5.b.v). pED935 contains a 23 kb PstI fragment from pED953 cloned in pBR325 and is

self-transmissible at approximately the same frequency as R46 (Ch. 5.b.v). The cloned DNA contains approximately 0.55 kb of Tn5 DNA, and the R46 DNA from between co-ordinates 13.45 and 35.95 kb. All the genes necessary for plasmid transfer are therefore located within this region of R46 (Fig. 6.7).

The R46::Tn5 plasmids pED959 and pED960 have Tn5 insertions at R46 co-ordinates 14.35 and 35.35 kb respectively, and are both Tra^+ (Fig. 6.7, Table 5.4). However, those 4 plasmids with insertions between co-ordinates 16.95 kb (pED954) and 31.2 kb (pED952) are Tra^- . The simplest interpretation of these data is that the conjugal transfer genes are all located between co-ordinates 14.35 and 35.35 kb in a region of between 14.2 kb and 21 kb in length.

Within this region the genetic determinants of certain transfer-related functions can be further localised. pED927, a recombinant clone of BglII-A confers sensitivity to the pilus-specific phages Ike and PR4, indicating that all the genes necessary for synthesis of the N pilus are between co-ordinates 21.4 kb and 35.35 kb (Fig. 6.7). Insertions of Tn5 at three different sites within this region of R46 abolish sensitivity to the two phages (pED958, 950 and 952; Fig. 6.7).

Since pED927 is Tra^- there must be essential transfer genes anticlockwise of the BglII site at co-ordinate 21.4 kb (Fig. 6.7) that are not required for synthesis of the conjugation pilus. These are most probably involved in conjugal DNA metabolism. The Tn5 insertion in pED954 (Fig. 6.7) presumably lies within one such gene since this plasmid is Tra^- but $\text{Ike}^S \text{PR4}^S$ (Table 5.4).

The genetic determinant of the surface exclusion property of R46 was localised by comparing the frequencies of transfer of the

Fig. 6.6 Physical and Genetic map of R46

The positions of the genetic determinants of plasmid phenotypes and functions are shown in relation to the restriction endonuclease cleavage map. Dotted lines indicate uncertainties in the map limits of genetic determinants (see text for details). The order of the six small PstI fragments around co-ordinate 40 kb was not determined. The position of the Tn5 insertion in pED953 is marked by the triangle, and the PstI fragment from pED953 cloned in pBR325 to construct pED935 is indicated. The figure also shows the region of R46 deleted in pKM101, the short inverted repeats flanking the Uvp and possibly Rep regions (see Fig. 4.3), and the two inverted repeat elements ISN(a) and ISN(b).

Key to restriction enzyme sites: R, EcoRI; H, HindIII, Bg, BglII; P, PstI; Sa, SalI; Ba, BamHI; K, KpnI; Sm, SmaI; X, XhoI.

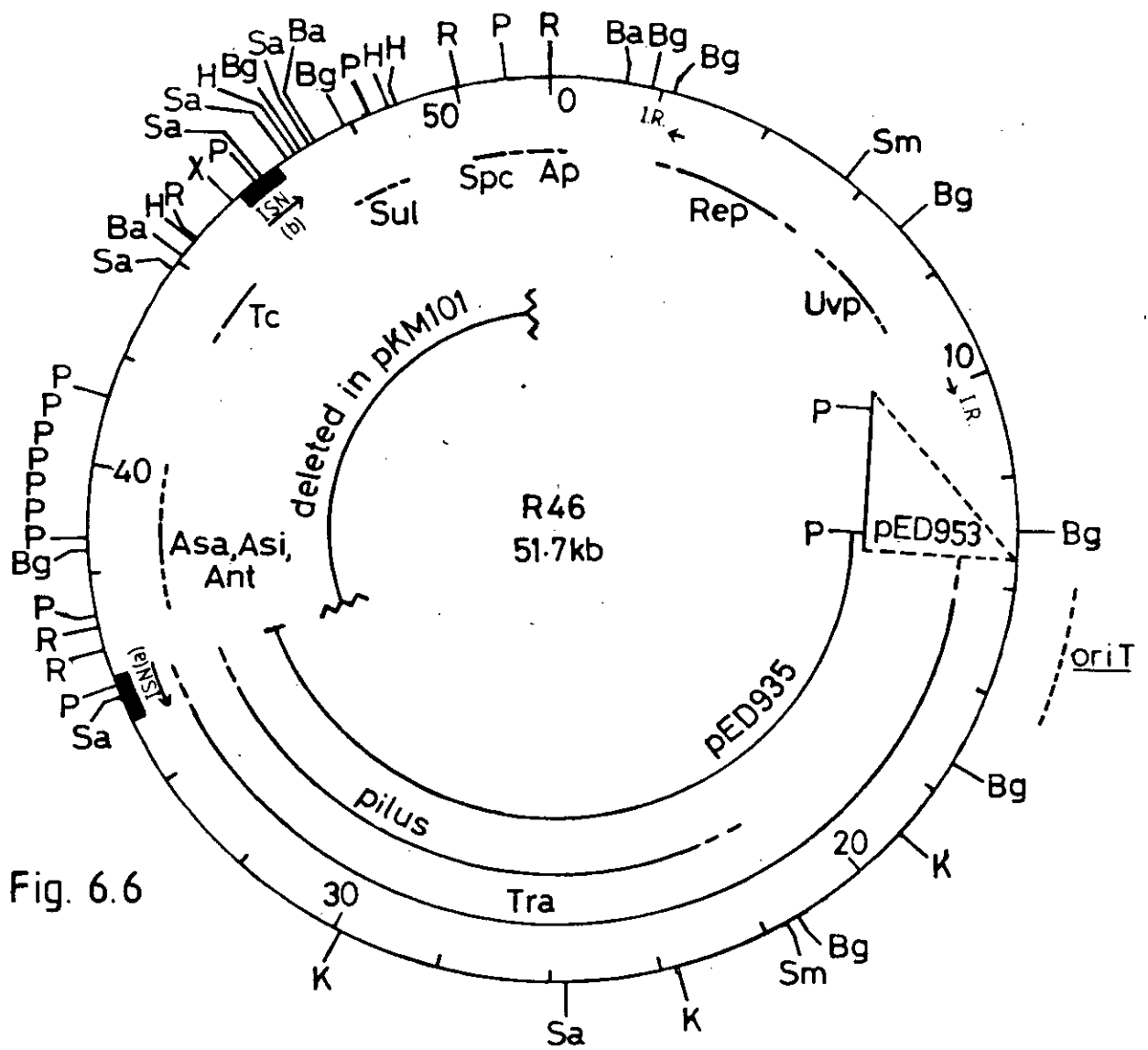


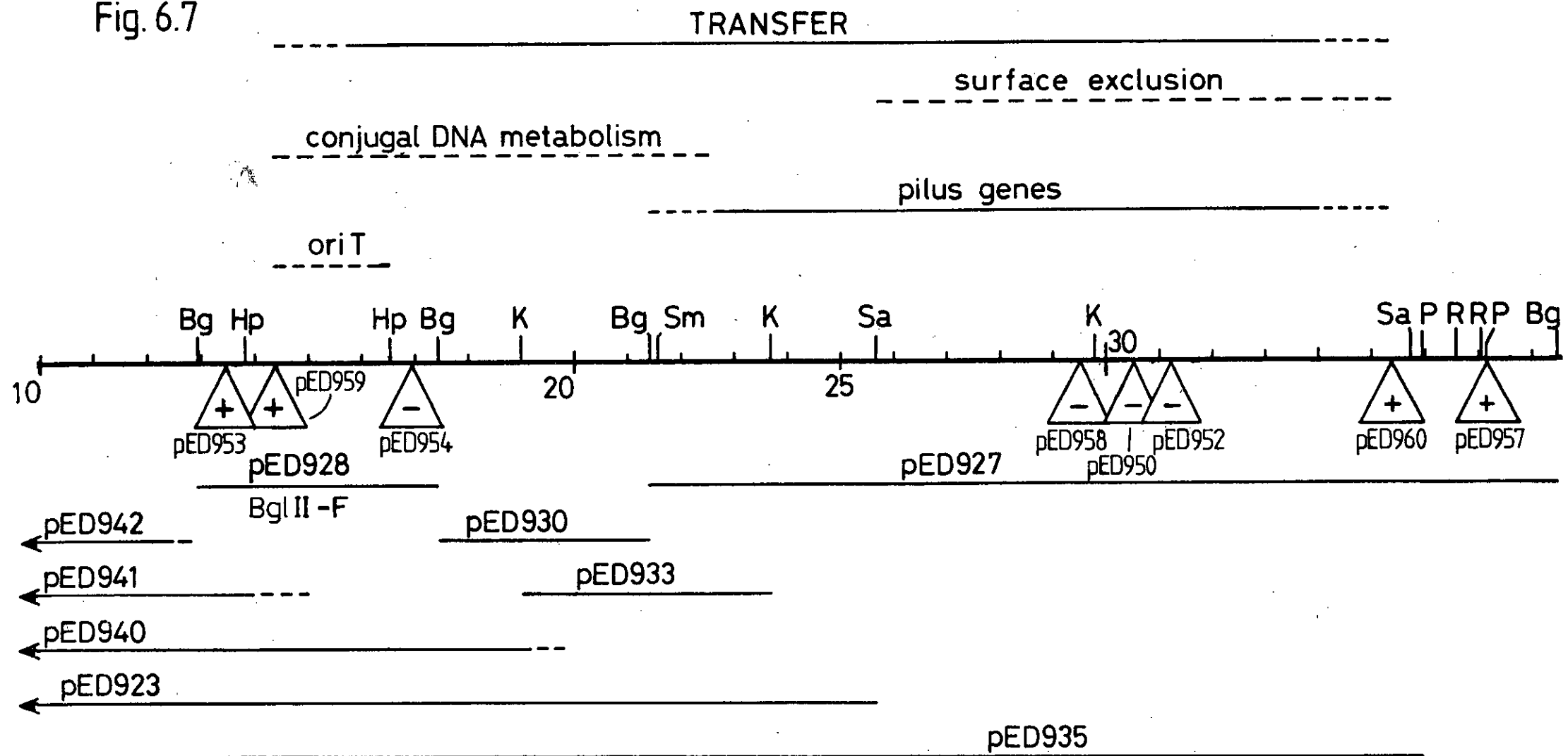
Fig. 6.6

Fig. 6.7

Expanded map of the transfer region of R46 showing the R46 DNA contained in relevant deletion and recombinant plasmids, and the approximate locations of the determinants of conjugal transfer functions (see text for details). Triangles mark the positions of the Tn5 insertions in the R46::Tn5 plasmids pED953, 959, 954, 958, 950, 952, 960 and 957. A '+' within the triangle indicates that the relevant plasmid is Tra⁺, and a '-' that it is Tra⁻. BglII fragment F (cloned in pED928) is marked; the location of oriT within this fragment is described in Chapter 8.

Arrowheads indicate that a plasmid extends off the figure. For key to restriction enzyme sites see legend to Fig. 6.6, and Hp = HpaI. Co-ordinates are marked in kilobases.

Fig. 6.7



Km^R Tra⁺ R46::Tn5 plasmid pED952 (Table 5.4) into recipient strains containing various R46 derivatives. The reduction in transfer frequency observed for Sfx⁺ plasmids in semi-quantitative matings (Ch. 2.c) was at least 100-fold. Preliminary data suggested that pED935 and pED927 were Sfx⁺, while pED923 was Sfx⁻. A region essential for surface exclusion may therefore lie between the SalI site at co-ordinate 25.7 kb and the PstI site at co-ordinate 35.95 kb (see Fig. 6.7).

(c) N3: Locations of Genetic Determinants on the Physical Map

(i) Replication region

The 5 in vitro deletion plasmids derived from N3 (Ch. 5.c.i; Table 5.5) have a single segment of the molecule in common, which extends from the EcoRI site at co-ordinate 0 kb to the BglII site at 11.9 kb (Fig. 6.8). This segment is also present in pED971 and pED972, the two deletion derivatives of pED902 that have been characterised (Ch. 5.c.ii; Fig. 6.10). Assuming that not more than one region of N3 can support autonomous replication, the 11.9 kb segment must contain DNA essential for this.

Moreover, restriction analysis of pED991, a spontaneous deletion derivative of N3 (Ch. 5.c.iii), indicates that the N3 DNA between co-ordinates approximately 52 and 5 kb is absent in this plasmid, each deletion end-point lying close to a PstI site (Fig. 5.5, Fig. 6.8). The essential replication region of N3 probably therefore lies between approximately co-ordinate 5 kb and the BglII site at co-ordinate 11.9 kb (Fig. 6.8).

Ando and Arai (1981) have recently suggested that N3 contains two distinct replication regions, each capable of supporting autonomous replication. Neither of these proposed regions lie

within the replication region defined above, so their results clearly disagree with those presented here. Restriction analysis of the N3 derivative pED972, however, showed that this plasmid contains neither of the replication regions proposed by Ando and Arai (op.cit.), while it does contain the segment from co-ordinates 5 kb to 11.9 kb (Fig. 6.10). This indicates that the location of the N3 replication region presented here is correct.

(ii) Antibiotic resistance determinants

Spectinomycin resistance

The in vitro deletion plasmid pED901 is composed of BglII fragments A and E of N3, and is Spc^R , whereas pED902, composed of HindIII-A alone, is not (Table 5.5). This indicates that the Spc^R determinant of N3 lies on BglII-E, at least partly between the HindIII site at co-ordinate 48.5 kb and the left-hand end of the BglII fragment as shown in Fig. 6.8. In addition pED914, an N3 derivative composed of EcoRI-A alone is Spc^S (Table 5.5); this positions an essential part of the Spc^R determinant between the HindIII site at co-ordinate 48.5 kb and the EcoRI site at co-ordinate 48.2 kb (see Figs. 6.8, 6.10).

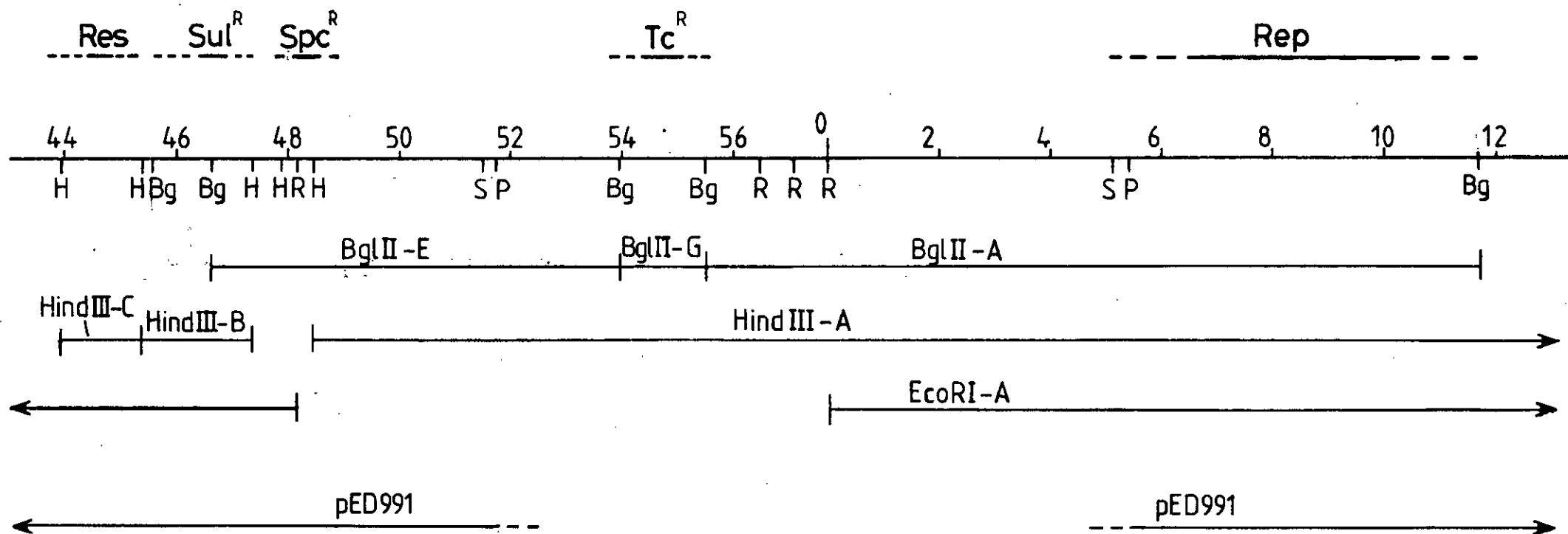
Sulphonamide resistance

The in vitro deletion derivatives pED908 and pED902 are composed of HindIII fragments A + B of N3, and HindIII-A alone, respectively (Table 5.5). pED908 is Sul^R and pED902 is not, implying that the presence of HindIII-B in pED908 imparts Sul^R to the plasmid phenotype. Moreover HindIII-A and B are not contiguous in the parent N3 molecule (Fig. 4.6), so the Sul^R determinant is probably located entirely within HindIII-B (Fig. 6.8). As predicted, pED914 (Table 5.6) which contains this fragment, is also Sul^R .

Fig. 6.8.

Expanded map of N3 between co-ordinates 43 kb and 12 kb showing restriction sites for EcoRI, HindIII and BglII (plus certain SalI and PstI sites). The location of the replication region (Rep) is indicated, as are the positions of the genetic determinants of tetracycline resistance, spectinomycin resistance, sulphonamide resistance, and the EcoRII restriction phenotype. The N3 DNA contained in the spontaneous deletion derivative pED991 is shown, and named restriction fragments relevant to the text are marked. Dotted lines indicate uncertainties in the map limits of phenotypic determinants (see text for details). Co-ordinates are marked every 2 kb. For key to restriction enzyme sites, see legend to Fig. 6.10.

Fig. 6.8



Tetracycline resistance

The Tc^R determinant of N3 must be located on HindIII fragment A since the Tc^R N3 derivative pED902 (Table 5.5) is composed of this fragment only. pED914, composed of EcoRI-A alone, does not determine Tc^R . At least one end of the Tc^R determinant probably therefore lies between N3 co-ordinates 48.5 kb (HindIII) and 0 kb (EcoRI) (see Fig. 6.8). All the DNA between these co-ordinates except that within BglIII-G is contained in pED901 (Table 5.5), but this plasmid is not Tc^R . An essential part of the Tc^R determinant of N3 therefore lies within BglIII-G (Figs. 6.8, 6.10).

The Tc^R phenotype of N3 is mediated by a Class AI Tc^R determinant (Mendez et al, 1980). This is related to the Class A Tc^R determinant of the IncP plasmid RP1 since the relevant gene products show similar substrate specificities, and a SmaI restriction fragment from the RP1 Tc^R determinant hybridises to N3 DNA (Mendez et al, op.cit.). The Tc^R determinant of RP1 contains two SmaI restriction sites and one SalI site (Grinsted et al, 1977; Depicker et al, 1977). From the restriction pattern in the Tc^R region of N3, however, it was not possible to identify a DNA segment homologous to the Tc^R determinant of RP1 (see Fig. 6.10). This indicates that the DNA sequences of the two determinants are substantially different.

(iii) EcoRII restriction phenotype (Res)

The genetic determinant of the EcoRII restriction endonuclease encoded by N3 was localised by comparing the titre of unmodified phage λ lysates, using host cells containing deletion derivatives of the plasmid. Table 5.5 shows that pED914 is Res^+ , while pED901,

902, 903 and 908 are all Res^- . pED914 is composed of EcoRI fragment A only so the EcoRII restriction determinant must lie within this fragment. The endonuclease is not produced in cells containing plasmids composed of HindIII fragments A and B (pED908) or BglII fragments A and E (pED901) (Table 5.5; Fig. 6.8). These data locate an essential part of the restriction determinant within the HindIII-C fragment of N3 (Fig. 6.8).

The position of the EcoRII modification methylene (Mod) determinant was not investigated, but is presumably within pED914 since this plasmid is Res^+ and a $\text{Res}^+ \text{Mod}^-$ phenotype would be lethal. By analogy with other type II restriction-modification determinants, the modification determinant is probably adjacent to that for the restriction endonuclease. Kosykh *et al* (1980) have cloned the EcoRII endonuclease and methylase genes of N3 on an 8.7 kb EcoRI restriction fragment, also containing the Sul^R determinant. A fragment of this size was not observed in the EcoRI digests of N3 DNA described in Chapter 4, nor in those reported by Ando and Arai (1981). However, the pattern of other restriction sites reported by Kosykh *et al* (1980) within the 8.7 kb EcoRI fragment is consistent with that shown in Fig. 6.8 for the EcoRII and Sul^R regions of the 48.2 kb EcoRI-A fragment of N3. The results of Kosykh *et al* (*op.cit.*) are most probably due to an extra EcoRI cleavage site in their isolate of N3 at approximately co-ordinate 39.5 kb in Fig. 6.10.

(iv) Conjugal transfer genes

The *in vitro* deletion plasmids pED902 and pED914 are composed of the HindIII-A and EcoRI-A fragments of N3, respectively (Table 5.5). Both plasmids are self-transmissible, indicating that all

the transfer genes of N3 are located between the EcoRI site at 0 kb and the HindIII site at 43.95 kb (Fig. 6.10). Within this large region there must be genes clockwise of approximately co-ordinate 24 kb that are necessary for plasmid transfer and N pilus synthesis, since pED971 is $\text{Tra}^- \text{Ike}^R$. This plasmid contains the N3 DNA from approximate co-ordinates 49 kb clockwise to 24 kb (Fig. 6.10).

Since the transfer genes of N3 are likely to share homology with those of R46, DNA heteroduplex experiments were carried out to estimate the extent of this homology, and to locate homologous regions. pED936 contains the complete R46 transfer region cloned in pBR325 (Ch. 5.b.v) and DNA heteroduplexes were prepared using a mixture of pED936 and N3 DNA. Prior to heteroduplexing, pED936 was made linear by digestion with HindIII, and N3 was digested with SmaI. This enzyme cleaves N3 into two fragments, one of which contains all but 0.55 kb of the plasmid (see Fig. 4.6).

Fig. 6.9 shows an example of the heteroduplex molecules photographed in the electron microscope. Only 2 complete, and 3 almost complete, molecules have so far been identified and measured.

The mean total length of double-stranded heteroduplexed DNA (regions C and H in Fig. 6.9) was 23.1 kb (± 0.85 kb), which agree well with the total length of R46 DNA present in pED936 (22.5 kb). Assuming N3 and pBR325 DNA do not form heteroduplex, N3 therefore contains DNA largely homologous to the entire transfer region of R46. (Under the conditions used, heteroduplex DNA will only form between strands with $>80\%$ sequence homology.) The R46 DNA in pED936 corresponding to region C in the heteroduplexes (Fig. 6.9) is not required for plasmid transfer (section b.iv above; Langer *et al*, 1981). This indicates that the transfer genes of N3 lie in

Fig. 6.9 Electron micrograph of a heteroduplex molecule formed between N3 and pED936.

Double stranded regions are indicated by the thicker lines in the diagram. M13 and ϕ X174 size standards are marked.

Note the stem-loop structure (E and F), and the extensive homology represented by region H.

<u>Region</u>	<u>Mean length/kb</u>	<u>Std. error/kb</u>	<u>Parent plasmid</u>
A	2.4	0.3	pED936
B	3.8	0.1	N3
C	0.7	0.05	both
D	2.6	0.2	N3
E	0.15	0.02	N3
F	1.05	0.09	N3
G	5.5	0.2	N3
H	22.4	0.8	both
I	3.4	0.4	pED936
J	21.4	0.5	N3

Lengths were estimated from measurements of 5 molecules, except those of regions I and J (3 and 2 molecules, respectively). Part of region J is missing in the molecule shown.

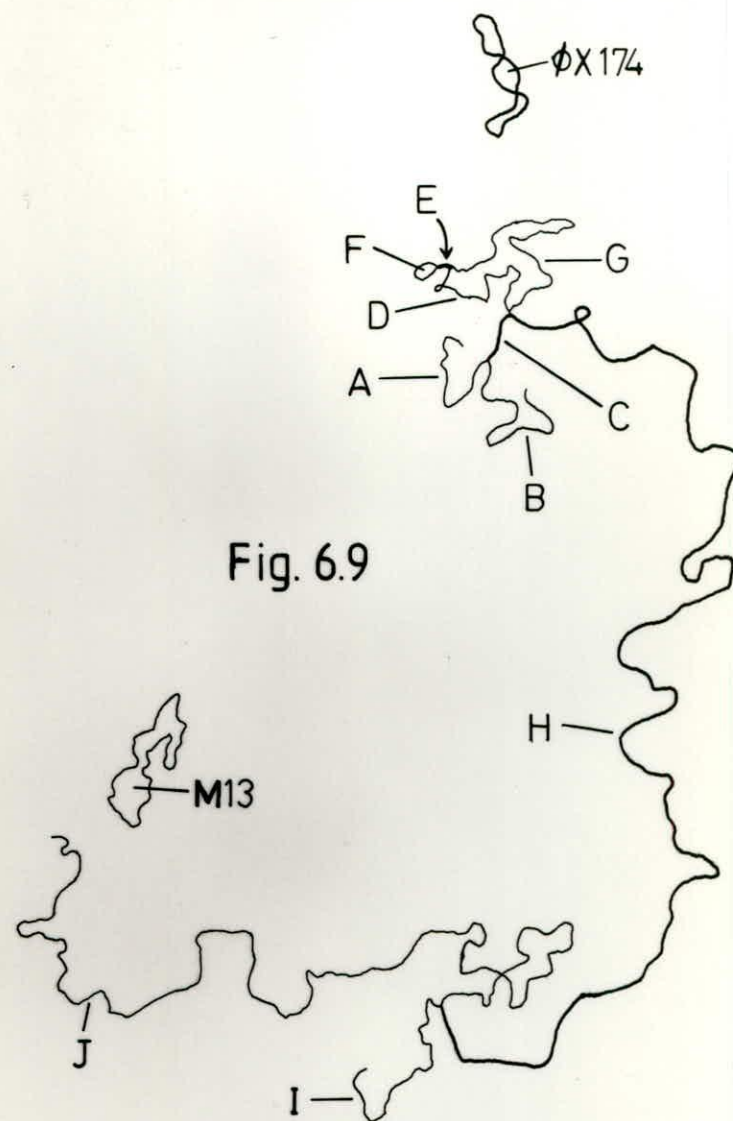
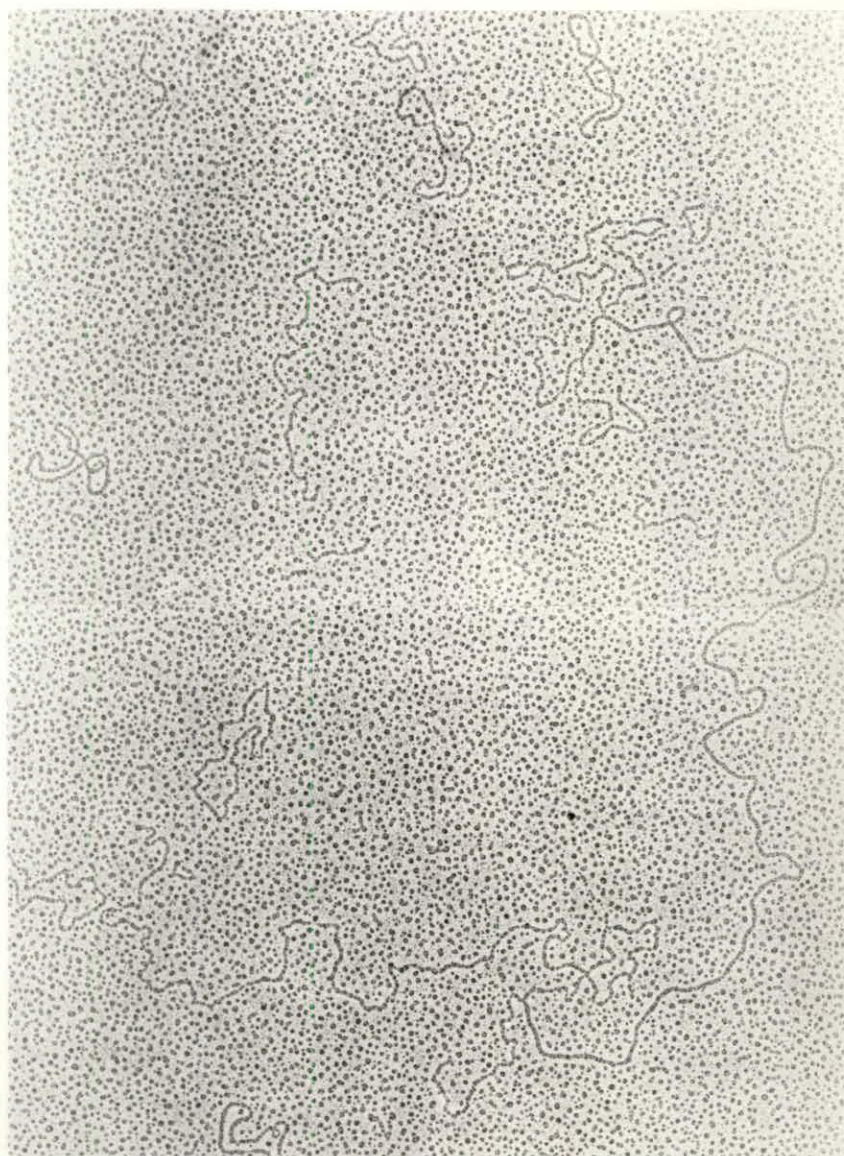


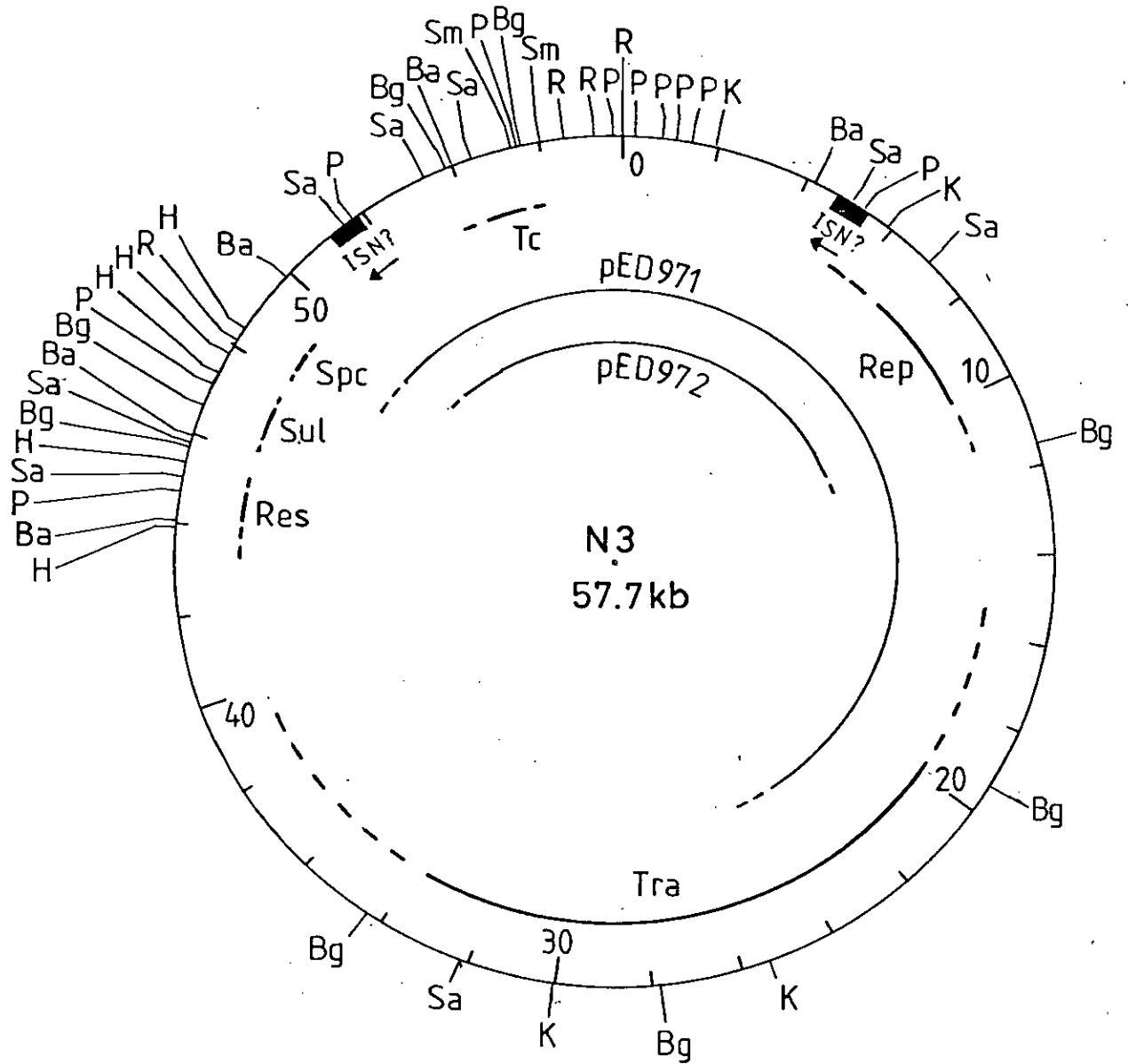
Fig. 6.9

Fig. 6.10 Physical and Genetic map of N3

The positions of the genetic determinants of plasmid phenotypes and functions are shown in relation to the restriction endonuclease cleavage map. Dotted lines indicate uncertainties in the map limits of particular determinants (see text for details). The location of the Uvp determinant of N3 was not investigated. The N3 DNA contained within the deletion derivatives pED971 and pED972 is indicated, and the possible positions of two directly repeated copies of the ISN element are shown. The segment marked Tra is that which heteroduplexes with the transfer region of R46.

Key to restriction enzyme sites: R, EcoRI; H, HindIII; Bg, BglII; P, PstI; Sa, SalI; Ba, BamHI; K, KpnI; Sm, SmaI.

Fig. 6.10



a single contiguous region of the plasmid equivalent to region H in Fig. 6.9. From the data available this region could be in either of two possible locations on the N3 restriction map and further experiments are required to resolve this ambiguity. The approximate map limits of these locations are indicated in Fig. 6.10.

Since all the R46 DNA in pED936 heteroduplexes with N3 DNA, the 9.3 kb (± 0.5 kb) insertion loop shown in Fig. 6.9 must be composed of N3 DNA. An interesting feature within this DNA is the small stem-loop structure (E and F in Fig. 6.9) indicative of a pair of closely spaced short inverted repeats. This structure was visible in 4 molecules out of 5. The length of the short duplex region was estimated as 150 bp (± 22 bp) and the loop size was 1050 bp (± 90 bp). The possible significance of this small stem-loop structure is considered in Chapter 7.

(d) Discussion

The combined physical and genetic maps of R46 and N3 are shown in Figs. 6.5 and 6.10, respectively. The locations of the genetic determinants on R46 are consistent with the Ap^R Uvp^+ Tra^+ phenotype of the R46 deletion derivative pKM101, given the map position of the deleted region (Ch. 4.c.ii; Fig. 6.5), and also with the map of pKM101 recently constructed by Langer et al (1981). Except for the location of the replication region, the data for N3 are in broad agreement with that recently obtained by Ando and Arai (1981).

The R46 DNA essential for plasmid maintenance and replication in E.coli is most probably located in a single region of less than approximately 3.4 kb. This region includes the R46 DNA corresponding to the approximately 2.5 kb segment recently identified by Langer et al (1981) as containing the replication region of pKM101.

Similarly the essential replication region of N3 is probably within a single 5.7 kb segment of the plasmid. No other genetic determinants have been mapped within these two regions of R46 and N3.

A number of other conjugative plasmids contain a short region of contiguous DNA sufficient to support autonomous replication, e.g. F (Lovett and Helinski, 1976) and R6-5 (Timmis et al, 1975), although replication of the broad host range IncP plasmid RK2 in E.coli requires two distinct segments of the molecule, which are separated by a Tc^R determinant (Thomas et al, 1980; C. Thomas, personal communication).

A single region of between 14.2 and 21 kb of R46 contains all the plasmid DNA necessary for conjugal transfer, and the whole of this region heteroduplexed with N3 DNA, indicating that the transfer genes of N3 are largely homologous to those of R46. Langer et al (1981) have recently identified the transfer region of pKM101 as a segment of between 14.4 and 15.7 kb in length; this corresponds to R46 DNA between co-ordinates 15.8 and 31.5 kb (see Fig. 6.7) and their result is thus consistent with those presented here for R46.

The two other conjugative plasmid transfer systems that have been most characterised to date are those of the IncF plasmids (e.g. F) and the IncP plasmids (e.g. RP4). The transfer system of the latter shows some similarities with that of IncN plasmids (see Chapter 1). The organisation of transfer genes in the IncF and IncP systems differ in that the F transfer genes lie in a single contiguous cluster of approximately 33 kb of DNA (Willetts and Skurray, 1980), whereas in RP4 the transfer genes are clustered in two or three distinct regions, two of which are separated by an antibiotic resistance determinant and ~15 kb of DNA (Barth et al, 1978; Barth 1979).

The transfer regions of R46 and N3 contain none of the other phenotypic determinants that were mapped above. The gene responsible for a plasmid-determined endonuclease (Lackey *et al*, 1977) has been located within the pKM101 transfer region (Langer *et al*, 1981; S.C. Winans, personal communication) but it is not yet known whether this gene product is required for transfer. The available data are therefore consistent with the organisation of the transfer genes of R46 and N3 in a single contiguous cluster on each plasmid. The length of plasmid DNA associated with R46 transfer functions, however, is approximately half that involved in transfer of the plasmid F.

The pattern of restriction sites in the regions of R46 determining Tc^R and Sul^R show striking similarities with the equivalent regions of other plasmids. As described above the restriction cleavage pattern of the Tc^R region was indistinguishable from that of the Class-C tetracycline resistance determinant of pSC101, and the Sul^R region appears to be similar to those of N3 and certain other plasmids. The Sul^R gene lies within a 1.4 kb segment of R46 bounded by BamHI and HindIII sites, and contains sites for BglIII and PstI. A similar pattern of BamHI-BglIII-PstI-HindIII sites is present in the HindIII fragment that includes the Sul^R determinant of N3, as well as in the Sul^R regions of the IncW plasmids R388 and Sa (Ward and Grinsted, 1978, and personal communication). It is therefore likely that these Sul^R determinants are homologous.

The overall distribution of restriction cleavage sites in both R46 and N3 is strongly asymmetric, and a large region of each plasmid contains many fewer sites than would be expected in random DNA sequences of the same length (Ch. 4.c.i, 4.d). These are the

regions containing DNA required for plasmid maintenance and replication, for conjugal transfer, and for the Uvp phenotypes. The majority of restriction sites present in R46 and N3 are located close to or within the regions responsible for antibiotic resistance phenotypes. A similar scarcity of cleavage sites in regions not associated with antibiotic resistance has been observed in plasmids of incompatibility groups W, P and Q (Ward and Grinsted, 1978; Barth, 1979; Thomas et al, 1980), which together with IncN plasmids are notable for their broad host-ranges. An irregular distribution of restriction sites may be a common feature among such plasmids. It has been suggested (Meyer et al, 1977; Barth, 1979; Thomas et al, 1980) that the phenomenon may be the result of selection for loss of cleavage sites in broad host-range plasmids, and that more numerous sites around the resistance determinants may reflect the relatively recent acquisition of these regions in the evolution of the plasmids. Transposition provides a plausible mechanism for the acquisition of resistance determinants, and evidence is presented in Chapter 7 that transposable elements are associated with the antibiotic resistance regions of R46 and N3.

Chapter 5.b.vi described the construction of a multicopy plasmid, pED981, containing the Uvp determinant of R46 cloned in pBR327, and a deletion derivative of this, pED985 (K. Singh and A. Brown, unpublished), allowed the R46 DNA sufficient for a Uvp⁺ phenotype to be located within a 3.1 kb segment. pED981 was initially constructed in order to test the hypothesis that a high copy number Uvp⁺ plasmid might enhance the level of chemically-induced mutagenesis beyond that determined by R46 or pKM101, and the properties of this plasmid merit further discussion.

Although pED981 conferred no greater protection than R46 against the lethal effects of UV irradiation, the plasmid caused a ten-fold increase in spontaneous and MMS-induced mutagenesis over and above the level determined by R46 (K. Singh and A. Brown, unpublished). A possible explanation of these differential effects on two of the Uvp phenotypes is that the R46 muc gene product is involved in two distinct cellular pathways, one conferring protection against UV-killing and the other causing enhanced mutagenesis. If these pathways had different rate-limiting steps, then increasing the cellular concentration of muc gene product might cause an increase in one phenotypic effect but not the other. There is no other evidence for two distinct pathways of this sort, however, and moreover it is widely believed that the repair of damaged DNA and the introduction of mutations are two manifestations of a single error-prone repair process (see Witkin, 1976).

An alternative explanation stems from consideration of the causes of death in UV-survival experiments. The major DNA lesion caused by UV-irradiation of cells is the formation of pyrimidine dimers (see Witkin, 1976). During DNA replication single strand gaps of about 1000 nucleotides in length are introduced opposite such lesions. Post-replicative recombination repair mediates the exchange of DNA between sister strands so that the gaps then lie opposite a template strand with no dimer in the complementary DNA sequence. The gaps are then repaired by polymerase activity.

Approximately 1 pyrimidine dimer per genome is introduced by 0.02 Jm^{-2} of UV (Howard-Flanders, 1968). In UV-survival experiments cells are irradiated with up to 100 Jm^{-2} so up to about 5000 dimers

per genome may be produced. The genome size of E.coli is approximately 4000 kb (Bachmann and Low, 1980) so under these conditions there is a high probability that cells will contain two adjacent pyrimidine dimers within 1 kb of each other in opposite DNA strands. Post replicational recombination^{tional} repair in such cells could lead to the appearance of overlapping gaps in opposite strands, and hence to double strand breakage. Such damage could not be repaired and is probably a major cause of death in UV-survival experiments.

One of the SOS responses induced by UV-irradiation is the failure of cell division, leading to filamentous growth (see Witkin, 1976). An additional cause of death in UV-survival experiments may be the severely reduced viability of filamenting cells plated on nutrient agar, relative to their viability in broth culture (Donachie and Hobbs, 1967; W. Donachie, personal communication). A proportion of the cells that die due to filamentation, however, will carry DNA damage that is essentially repairable. An increased rate of cellular DNA repair activity could therefore rescue these cells from death.

When R46 is present in a cell population, the additional repair capacity imparted by the plasmid may be sufficient to allow repair of virtually all the UV-induced DNA damage that is potentially repairable. The resulting UV-survival graph would then be a reflection of the number of cells which had suffered irreparable damage (such as that caused by closely adjacent pyrimidine dimers) and survival would be unaffected by a further increase in the cell's capacity for DNA repair. This would explain the observation that pED981, although present in higher copy numbers than R46, does not

confer additional UV-protection over and above the level determined by R46. Under the conditions used in MMS mutagenesis experiments, however, no significant cell death occurs (Ch. 2.e.iii), indicating that virtually all the induced DNA damage is potentially repairable. An increased ratio of error-prone to error-free DNA repair, resulting from additional copies of plasmid-borne muc genes, would therefore be expected to produce a further enhanced level of MMS-induced mutagenesis, as was observed for cells containing pED981.

The differential effects of pED981 on UV-survival and MMS-induced mutagenesis can therefore be reconciled with the concept that both these phenotypic effects are manifestations of one and the same error-prone repair pathway. The greatly enhanced level of chemically-induced mutagenesis determined by pED981 may permit the development of a more sensitive test for the identification of carcinogens as mutagens in bacteria. Experiments are in progress to establish whether the properties of pED981 are similar in the strains of Salmonella typhimurium routinely used for such tests.

CHAPTER 7. TRANSPOSABLE ELEMENTS ON R46 AND N3

(a) Introduction

Transposable elements can be divided into two classes on the basis of their genetic complexity: insertion sequences and transposons (Campbell et al, 1977). Insertion sequence (IS) elements can be as small as 768 bp (Ohtsubo and Ohtsubo, 1978) and carry no identifiable phenotypic determinant, while transposons are generally larger than 3 kb, and include genes determining specific phenotypes, such as resistance to particular antibiotics (see Calos and Miller, 1980). Both classes of element are closely related, however, and both are commonly found on bacterial plasmids.

'Direct' transposition is manifested by the appearance of a new copy of a transposable element within a DNA sequence on which it was not previously located. Transposable elements can also cause other genetic rearrangements, most of which are consequences of the transposition mechanism. These include replicon fusion, the deletion, inversion, and translocation of adjacent DNA segments, and precise excision of the elements themselves (for reviews see Kleckner, 1977; Starlinger, 1980; Calos and Miller, 1980). The probable importance of such DNA rearrangements and translocations in plasmid evolution has been briefly discussed in Chapter 1.

A characteristic feature of transposable elements in prokaryotes (with the exception of bacteriophage Mu (Kahmann and Kamp, 1979)) is the presence of inverted repeat sequences at their termini. For insertion sequences and some transposons, such as Tn₃, these are typically 20-40 bp in length, while in other transposons, such as Tn₅ and Tn₁₀, they are considerably longer (1000-1500 bp). The latter class of transposons are compound structures that include

indirectly repeated copies of IS or IS-like elements at their termini. Other compound transposons are bounded by directly repeated copies of insertion sequences. In the case of Tn₉ these are two copies of IS₁, and Tn₉ therefore has short terminal inverted repeats identical to those of IS₁. The transposition of these compound transposons appears to be mediated entirely by the flanking IS-elements. For example the terminal inverted repeats of Tn₅ and Tn₁₀, named IS₅₀ and IS₁₀ respectively, are themselves capable of transposition and encode all the functions necessary for transposition of the compound Tn element (Rothstein *et al*, 1981; Foster *et al*, 1981). It seems probable that any DNA segment flanked by copies of an insertion sequence in either orientation is potentially transposable.

The transposition mechanism most extensively studied to date is that of the Ap^R transposon Tn₃, which does not contain IS-like elements, and which encodes gene products required for its own transposition (see Heffron *et al*, 1981). Transposition of this element between replicons occurs via an obligatory intermediate step in which the 'donor' and 'target' replicons are fused as a cointegrate, with two directly repeated copies of the transposon at the inter-replicon boundaries (Gill *et al*, 1978; Arthur and Sherratt, 1979). Tn₃ encodes a *recA*-independent recombination enzyme which rapidly resolves these cointegrates into the transposition products normally observed, by cleavage and religation at specific sites within each copy of the transposon (Arthur and Sherratt, 1979; Kostriken *et al*, 1981; Reed and Grindley, 1981). In the light of these data Shapiro (1979) and Arthur and Sherratt (1979) proposed models for transposition which account for many of

the observed properties of Tn3 and a group of elements closely related to it.

Insertion sequences, however, and compound transposons containing IS elements, probably do not encode site-specific recombination systems analogous to that of Tn3, as is implied by the relative stability of elements like Tn9. Moreover, while replicon fusion is a common property of IS elements, Galas and Chandler (1981) and Grindley and Joyce (1981), found that cointegrates produced by elements such as IS1, Tn5 and IS903 are relatively stable. In view of this stability, and the frequency with which 'direct' transposition of these elements is observed, these authors concluded that an alternative pathway exists for transposition, that does not involve obligatory cointegrate intermediates. Galas and Chandler (op.cit.) proposed a general model suggesting two possible pathways for IS-mediated transposition: one resulting in 'direct' transposition, and the other in cointegrate formation. Whilst such cointegrates may resolve by recA-mediated recombination, in the absence of an efficient site-specific mechanism they will be relatively stable, especially when formed by small insertion sequences.

Whether or not cointegrate formation is a necessary intermediate in their transposition, all transposable elements can probably mediate replicon fusions, and this property can be exploited in the search for novel plasmid-borne insertion sequences and transposons, by using a conjugational mobilisation method to select replicon fusion products. Non-conjugative plasmids can be mobilised at low frequencies by conjugative plasmids as part of a cointegrate formed by a transposable element on the latter molecule (Guyer, 1978; Crisona et al, 1980; Willetts et al, 1981). If such cointegrates

are subsequently resolved, the resulting non-conjugative plasmid derivative, containing an insertion of the transposable element, facilitates physical and genetic characterisation of that element. A cointegrate selection method was consequently adopted in experiments designed to detect transposable elements on R46 and N3.

(b) Detection of Transposable Elements on R46

(i) Transpositional mobilisation of pED815

The $\text{Cm}^R \text{ Tc}^R$ plasmid pED815 (Johnson and Willetts, 1980) is a 5.75 kb mobilisation deficient (see Chapter 8.a) Ap^S deletion derivative of pBR325, which contains no known intact transposable elements. pED815 was used as a vector or 'target' replicon for transposable elements on R46, and transposition-mediated cointegrates of the two plasmids were selected by conjugational mobilisation of the Cm^R determinant of pED815.

Donor cultures derived from single colonies of the recA^- strain JC6310 (R46, pED815) were crossed with the Nal^R strain ED3818 in membrane filter matings, and both $\text{Cm}^R [\text{Nal}^R]$ and $\text{Spc}^R [\text{Nal}^R]$ transconjugants were selected. The frequency of mobilisation of the Cm^R marker was 5×10^{-7} relative to the transfer frequency of R46, and all the Cm^R transconjugants tested carried the antibiotic resistance phenotypes of both plasmids. In order to establish whether these cells contained R46::pED815 cointegrates or two distinct plasmids, ED8654 was transformed with plasmid DNA prepared from two Cm^R transconjugants, and Cm^R transformants were selected. In one case all the transformants recovered had the phenotype of pED815 alone, and were subsequently shown to contain plasmids indistinguishable in size from pED815. The mechanism by which these plasmids were mobilised is not clear, but may possibly involve

low-frequency recognition of an oriT-related sequence (Chapter 8) on pED815. In the other case, however, all 30 transformants tested were $\text{Cm}^R \text{ Tc}^R \text{ Ap}^R \text{ Spc}^R \text{ Sul}^R$, suggesting that the markers were present in an R46::pED815 cointegrate, since the co-transformation frequency of two plasmids is low. This plasmid was numbered pED970, and its cointegrate structure was confirmed by restriction analysis (Fig. 7.2). pED970 contains all the BglII fragments of R46, excepting BglIII-B, which is increased in size by approximately 7 kb. This indicates that pED815, which contains no sites for BglII, has integrated within BglIII-B. BglIII-B of R46 contains ISN(b), one of the inverted repeat sequences demonstrated in Chapter 4, which may perhaps be insertion sequences. If pED970 had been formed as a result of ISN(b) transposition, the cointegrate would be predicted to contain two copies of this element, repeated on either side of the pED815 insertion, in direct orientation (see Fig. 7.1), and the SalI and PstI sites within ISN(b) (Fig. 4.2) should therefore be duplicated. pED815 contains one site for SalI and none for PstI, so the restriction cleavage patterns of pED970 DNA shown in Fig. 7.2 confirm these predictions. pED970 contains all the PstI fragments of R46, as well as an extra fragment corresponding to the length of the pED815 insert plus the duplicated transposable element (Figs. 7.1, 7.2). The cointegrate also contains all the SalI digestion products of R46, plus two extra fragments, resulting from the SalI site in pED815 and that in the duplicated copy of ISN(b). Comparison of the sizes of the novel SalI and PstI restriction fragments with that of pED815 allowed the length of the duplicated transposable elements to be estimated as between 0.7 and 1.0 kb, which agrees well with the length of

Fig. 7.1

Predicted structure of an R46::pED815 cointegrate formed by transposition of ISN(b) into pED815, compared with that of R46. Only the region between R46 co-ordinates 44 and 48 kb is shown, and only the restriction sites for PstI, SalI and BglII are marked. Part of BglII fragment B, which extends off the figure, is shown. pED825 DNA is indicated by the thicker line. ISN(b) is duplicated in the transposition event, so that the pED815 DNA in the cointegrate is flanked by directly repeated copies of the element. Since each element contains a site for PstI and for SalI, all the PstI and SalI fragments of R46 will be present in the cointegrate, together with the novel fragments indicated above the map. The size of the novel PstI fragment, and the sum of the sizes of the two novel SalI fragments, each represent the length of pED815 plus the length of one copy of ISN(b). As there is no BglII site within ISN(b) or pED815, the size of BglII-B will be increased in the cointegrate, and no novel BglII fragments will be present. Fig. 7.2 shows that the structure of pED970 is similar to that shown for the cointegrate in this figure.

Key to restriction enzyme sites: P, PstI; Sa, SalI; Bg, BglII.

Fig.7.1

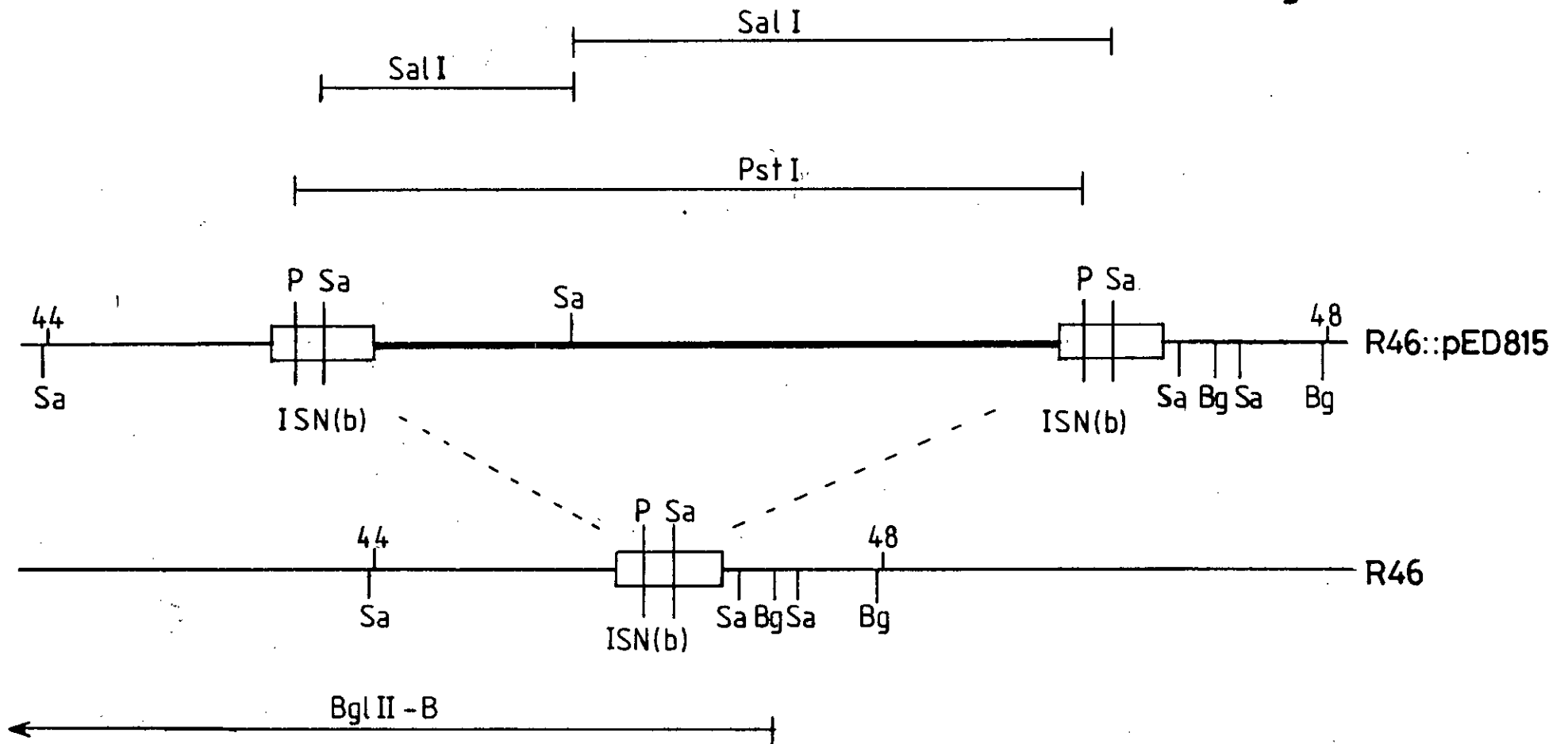


Fig. 7.2

Restriction enzyme cleavage analysis of the R46::pED815 cointegrate plasmid pED970, using 0.7% agarose gel electrophoresis.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	R46	<u>BglII</u>
2	pED970	<u>BglII</u>
3	λ 7	<u>HindIII</u>
4	R46	<u>PstI</u>
5	pED970	<u>PstI</u>
6	pED815	<u>HindIII</u>
7	λ 7	<u>HindIII</u>
8	R46	<u>SalI</u>
9	pED970	<u>SalI</u>
10	pED815	<u>SalI</u>

- Notes:
- i) Comparison of tracks 1 and 2 shows that pED970 contains an insertion within BglII fragment B.
 - ii) One extra PstI fragment and two extra SalI fragments are visible in tracks 5 and 9, in addition to the full complement of R46 fragments (tracks 4 and 8). A PstI and a SalI site have therefore been duplicated in formation of the cointegrate (pED815 contains one SalI site).
 - iii) The sizes of HindIII fragments of λ 7 DNA and of their pED815 DNA are marked in kilobases.

Fig. 7.3.

Restriction analysis of pED994 (pML31::ISN) using 0.7% agarose gel electrophoresis.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	λ 7	<u>HindIII</u>
2	pML31	<u>PstI</u>
3	pED994	<u>PstI</u>
4	λ 7	<u>HindIII</u>
5	pED994	<u>EcoRI</u>
6	pML31	<u>EcoRI</u>

- Notes:
- i) pED994 contains one more PstI fragment than pML31, due to a PstI site in the inserted DNA.
 - ii) Tracks 5 and 6 show that pED994 is approximately 0.9 kb larger than pML31.

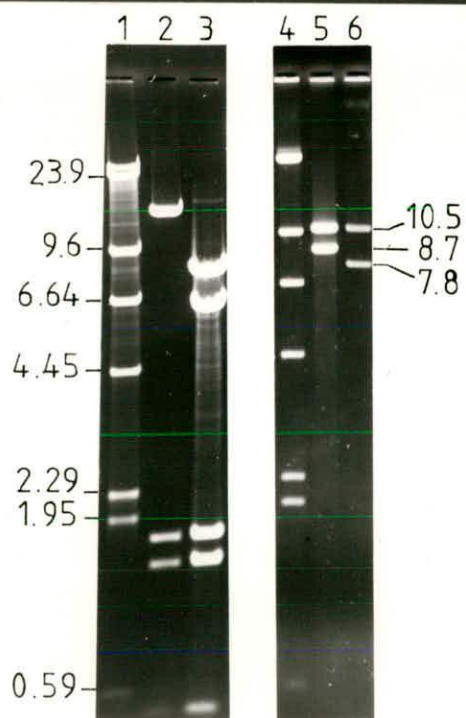
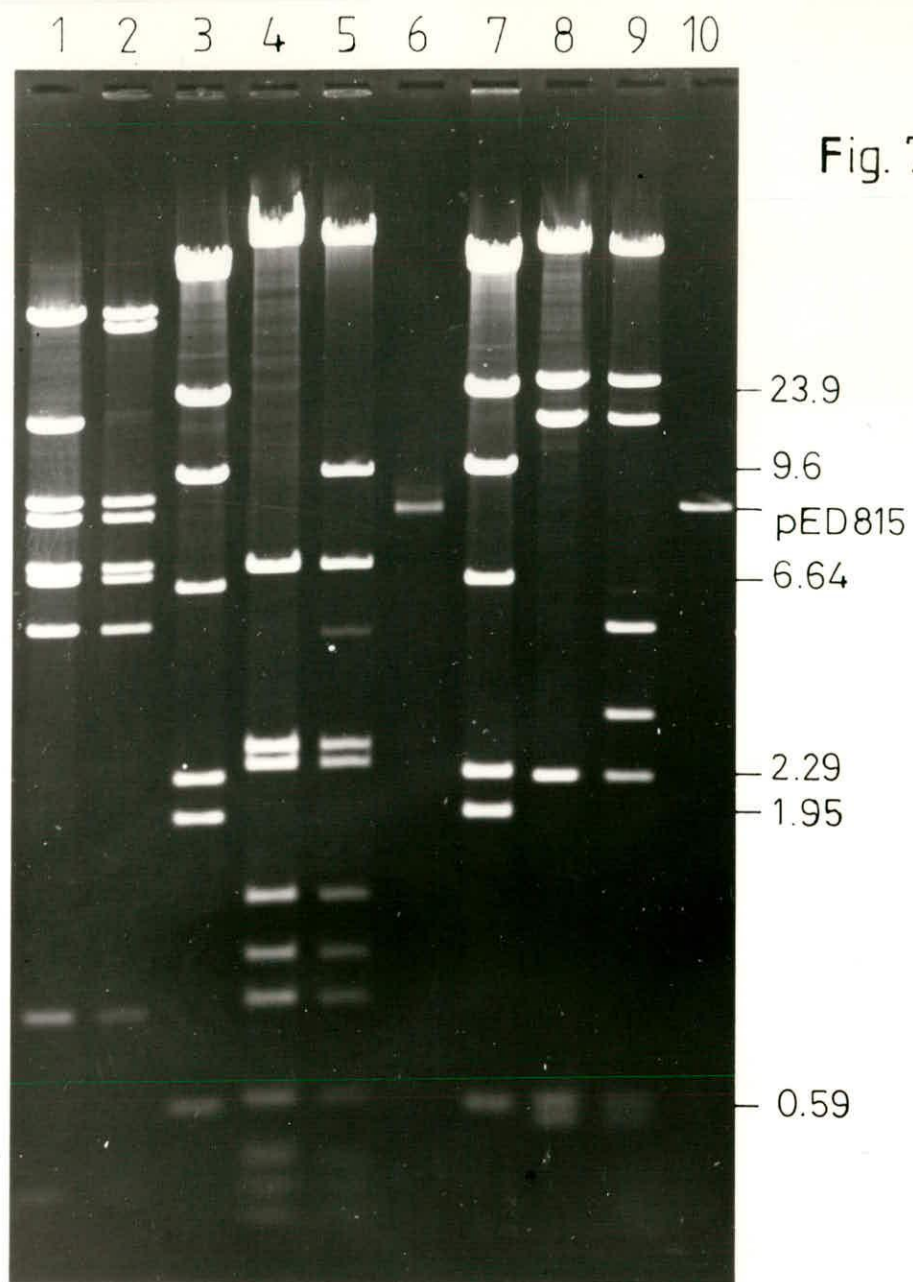


Fig. 7.3

ISN(b) measured by electron microscopy (865 bp $\pm$ 30 bp, Ch. 4.ciii). The structure of pED970 is therefore that predicted for a cointegrate molecule formed by transposition of ISN(b) from R46 into pED815 (see Fig. 7.1). Although only one such cointegrate has so far been characterised, this is strong evidence that ISN(b) is indeed a transposable insertion sequence. A second putative R46::pED815 cointegrate, pED996, generated in a separate experiment, had suffered a deletion in the vicinity of the ISN elements and was not further characterised.

Although pED970 was apparently stable it should potentially be resolved in rec⁺ strains into R46 and pED815::ISN(b) by homologous recombination. Since such recombination is likely to occur at low frequency, however (perhaps less than 10^{-6} per generation), attempts were made to select the predicted pED815::ISN(b) breakdown product of pED970 by taking advantage of the Slo phenotype of the latter plasmid (see Ch. 3.b.iii). After growing ED8654 (pED970) through approximately 80 generations, plasmid DNA was prepared and used to transform ED8654. Cm^R transformants were then selected on minimal agar plates so that colonies transformed with pED970 would grow slowly, while any containing pED815::ISN(b) would be Slo⁻, and so appear earlier on the selective medium. 34 such fast growing colonies were obtained among approximately 10^4 transformants but all were Cm^R Tc^R Ap^R Spc^R, and 3 investigated further were Ike^R. Since the Slo determinant of R46 lies adjacent to the pilus genes, at approximately co-ordinate 34 kb (Langer et al, 1981; see Fig. 6.6), this suggests that the fast growing transformants contain pED970 derivatives from which the Slo determinant (and in some cases pilus genes) had been deleted, rather than the desired pED815::ISN(b)

✓ plasmid. If this interpretation is correct, Slo^- deletions are generated from pED970 at a frequency of approximately 10^{-5} - 10^{-4} per generation. However, no firm conclusion can yet be drawn concerning the frequency of resolution of ISN-mediated R46::pED815 cointegrates by recombination, since the data so far available for pED970 cannot exclude the possibility that the ISN element is inserted within the replication region of pED815. If this were the case, the desired pED815::ISN plasmids would never be recovered in the above procedure. These experiments clearly need to be repeated with a number of independently isolated R46::pED815 cointegrates formed by insertion of ISN at different sites within pED815.

(ii) Mobilisation of R46 markers by R388

Preliminary experiments were conducted using a modified version of the transpositional mobilisation method described above, in which the non-conjugative plasmid was pED889 (a Tra^- point mutant of R46), and the conjugative plasmid was R388 (Sul^R Tp^R , IncW), which carries no known transposable elements. JC6310 (pED889, R388) was crossed with ED3818 in filter matings and Ap^R , Spc^R and Tc^R transconjugants separately selected. In this procedure the Tra^+ plasmid R388 was the 'target' for transposition, and in addition to transposition-mediated cointegrates, this should allow detection of 'direct' transposition products, i.e. R388 derivatives carrying insertions of discrete antibiotic resistance transposons.

In view of the conclusions reached by Galas and Chandler (pers. com., see P.91) this technique may be more efficient than that described in section (i) at detecting transposable elements that cause insertions by direct transposition more frequently than they form cointegrates. The

transconjugants obtained in these experiments have not yet been fully characterised, but preliminary data suggest that at least two R388::pED889 cointegrates were produced, the points of integration lying within BglIII-B of R46 in each case, and at two different sites in R388. These cointegrates were probably generated by transposition of ISN(b). In addition an $\text{Ap}^R \text{Spc}^R$ R388 derivative was recovered that carried an insertion of approximately 3 kb of pED889 DNA containing the Ap^R and Spc^R determinants. Yamamoto et al (1981a) have recently alluded to an Ap^R transposon on R46, and the 3 kb segment detected above may correspond to this element. Further experiments are required to confirm this, however.

(c) Detection of Transposable Elements on N3

The technique employed in these experiments was analogous to the transpositional mobilisation method described in section b(i) above, except that the conjugal crosses were initially performed with a rec^+ donor strain. The 'target' replicon used was pML31 (Timmis et al, 1978), a non-transmissible Km^R mini-F replicon. pML31 has previously been used in Tn3-mediated mobilisation experiments by Crisna et al (1980), although this plasmid itself carries the Km^R transposon Tn903 (Nomura et al, 1978), which is transposable at low frequency.

ED395 (N3, pML31) was mated with ED3818 and Km^R transconjugants were recovered at a frequency of 10^{-6} relative to N3 transfer. In one experiment the 6 transconjugants obtained were $\text{Km}^R \text{Spc}^R \text{Sul}^R \text{Tc}^R$, and all 4 phenotypes were co-transferred in subsequent conjugal crosses. Agarose gel electrophoresis of cleared lysate preparations showed that the 6 transconjugants contained plasmids larger than N3,

Table 7.1 Mobilisation of pML31 and pED994 by N3

<u>Donor Strain</u>	<u>Mean Transconjugant Frequency</u>		<u>Mobilisation Frequency of Km^R determinant</u>
	<u>Spc^R</u>	<u>Km^R</u>	
ED395 (N3, pML31)	2.4	2.8×10^{-6}	1.2×10^{-6}
ED395 (N3, pED994)	1.1	6.9×10^{-4}	6.3×10^{-4}
JC6310 (N3, pML31)	3.2	8.7×10^{-6}	2.7×10^{-6}
JC6310 (N3, pED994)	3.3	2.5×10^{-3}	7.6×10^{-4}

Data is from 30 min membrane filter matings. Transconjugant frequencies are frequencies per donor cell.

Mobilisation frequency of Km^R is expressed in relation to tranfer of Spc^R

ED395 is rec⁺, JC6310 is recA56; the recipient strain was ED3818.

and no other plasmids were visible. Based on the assumption that these were N3::pML31 plasmids formed by transposition of an element on N3, attempts were made to isolate cointegrate resolution products by P1 transduction, a method successfully used in analogous experiments by Willetts et al (1981). ED395 was transduced with P1 lysates grown on 3 of the Km^R transconjugant colonies, and Km^R transductants were selected. 186 of these were screened for N3-determined phenotypes and 185 were Km^R Spc^R Sul^R Tc^R. The remaining one was resistant to Km only, and contained a plasmid numbered pED994. Restriction analysis of this plasmid showed it to be a pML31 derivative, containing an insertion of approximately 0.9 kb of DNA (Fig. 7.3). This suggested that the inserted segment might be related to the 865 bp ($\pm$ 30 bp) ISN element of R46 shown above to be transposable. Both ISN elements on R46 contain single restriction sites for SalI and PstI (Fig. 4.2), and restriction analysis demonstrated that the inserted segment in pED994 also contains a site for each of these enzymes (Fig. 7.3, and data not shown). The restriction data in Chapter 4 suggested that N3 might contain two copies of ISN elements similar to those on R46, and pED994 is most probably the result of transposition of one such element onto pML31. Although the initial transposition product was an N3::pML31 cointegrate this was apparently resolved at low frequency to produce pED994.

In order to determine whether the presence of the inserted DNA in pED994 has any effect on the mobilisation of the plasmid by N3, the mobilisation frequencies of pED994 and pML31 were compared in membrane filter matings. Table 7.1 shows that pED994 is mobilised by N3 at least 200 times more frequently than pML31, from both

rec⁺ and rec⁻ donors. The reasons for increase mobilisation in the recA⁻ background are not clear, but perhaps the increased number of ISN elements in the cells containing pED994 lead to a disproportionate increase in the frequency of cointegrate formation by transposition.

Attempts to generate further pML31::ISN plasmids by the above method were discontinued following evidence that transposition of the Tn903 element on pML31 was detectable at frequencies comparable to ISN-mediated cointegrate formation. These experiments should now be repeated using a different target replicon for transposition, such as pBR322.

(d) Discussion

Data presented in Chapter 4 demonstrated that R46 contains a pair of 865 bp (± 30 bp) inverted repeat sequences, designated ISN(a) and ISN(b), which each contain single restriction sites for SalI and PstI (see Fig. 4.2). Furthermore the presence of two pairs of closely spaced sites for the same enzymes on N3 suggested that this plasmid also may contain two copies of the ISN element, although these would be in direct orientation. The experiments described in this Chapter are incomplete, but nevertheless provide strong evidence that ISN elements on R46 and N3 are transposable. The structure of the R46::pED815 cointegrate pED970 is precisely that expected for a product of replicon fusion mediated by transposition of ISN(b). Attempts to isolate the pED815::ISN(b) resolution product of this cointegrate have so far been unsuccessful, but the stability of pED970 is not surprising since cointegrates generated by known short insertion sequences are also stable even in rec⁺ hosts

(Ohtsubo *et al*, 1981; Grindley and Joyce, 1981). Moreover for IS-mediated transposition, stable cointegrates may be final products of the transposition process, as an alternative to simple insertions of the element in the target replicon (Galas and Chandler, 1981). Preliminary evidence was also obtained that ISN(b) can cause replicon fusions between an R46 derivative and R388 at at least two different sites of cointegration in the latter plasmid. From these results taken together, therefore, it seems reasonable to conclude that ISN(b) is an active insertion sequence.

Comparisons of the length and restriction cleavage pattern of ISN(b) with equivalent data for known insertion sequence indicates that ISN(b) is distinct from IS₁, IS₂, IS₃, IS₄, IS₅, IS₁₀, IS₂₁, IS₅₀ and IS₉₀₃ (Ohtsubo and Ohtsubo, 1978; Ghosal *et al*, 1979; Sommer *et al*, 1979; Habermann *et al*, 1979; Blattner *et al*, 1974; Kleckner, 1979; Willetts *et al*, 1981; Rothstein *et al*, 1980; Oka *et al*, 1981), and that ISN(b) is probably therefore a hitherto undescribed transposable element.

The structure of the cointegrates produced in transpositional mobilisation experiments with N3 and pML31 was not analysed, but in this case a presumptive cointegrate breakdown product was isolated. This plasmid, pED994, is comprised of pML31 with an insertion of approximately 0.9 kb of DNA containing single cleavage sites for SalI and PstI. In view of the similarity between this inserted segment and the transposable ISN(b) element on R46, pED994 probably results from the transposition of an ISN element on N3. Analysis of other transposition products, and of the 'parental' N3::pML31 cointegrate is required to confirm this, however, and to determine which copy of ISN has transposed. Also DNA hybridisation or hetero-

duplex studies are needed to demonstrate the presumed homology between the ISN elements on R46, the N3 DNA present in pED994, and the two regions of N3 suggested above to contain ISN elements.

Although direct evidence has only been presented for the transposition of one ISN element on each of R46 and N3 the extensive homology between ISN(b) and ISN(a) on R46 (Ch. 4.c.iii) suggests that the latter element may also be transposable. Similarly both presumptive copies of ISN on N3 may be transposable.

One of the properties manifested by insertion sequences is the deletion of adjacent segments of DNA. Such deletions have one end-point at the last nucleotide of the transposable element and extend outwards for variable distances, so that the element itself is not deleted (see Starlinger, 1980). Deletions of this sort generated from ISN(a) have already been implicated in the formation of the R46 deletion derivative pKM101 (Ch. 4.e), and if, as suggested, R46 and N3 each contain a pair of ISN insertion sequences, the origin of certain other in vivo deletion derivatives identified in the course of this study may be explained in a similar manner.

The deletion plasmids pED973 and pED974 were produced from R46 in ED575 (R46), a strain containing Tn₅ (Ch. 5.b.iv). It is possible that the deletions in these plasmids were generated by Tn₅ following insertion of the transposon, but BglIII restriction analysis showed no evidence of Tn₅ or IS₅₀ DNA in either plasmid, and when pED973 and pED974 were introduced into ED395 by transformation, both resulting strains were sensitive to kanamycin. At least one end-point of the deleted region in each plasmid lies within one of the BglIII fragments of R46 that includes an ISN element, however,

suggesting that the deletions may have been generated by one such element. Preliminary data is compatible with this interpretation, but further mapping of the deletion end-points is required to confirm it. It is tempting to speculate that the apparently high frequency of deletions formed in ED576 (R46) may reflect an interaction between Tn5 gene products and the ISN elements of R46.

The two spontaneous Tc^S deletion derivatives of N3 described in Ch. 5.c.iii, pED990 and pED991, may be further examples of plasmids formed by ISN-mediated deletion. Again further data are required to confirm this, but both deletions have an end-point within the PstI fragment C of N3, a fragment bounded by one of the putative ISN repeats (Fig. 5.5, Fig. 7.4). Moreover, PstI restriction analysis of pED991 shows no novel sized PstI fragments, and indicates that both end-points of the relevant deletion lie close to the proposed locations of ISN elements in N3 (Fig. 5.5, see Fig. 7.4). If, as suggested above, the ISN repeats in N3 lie in direct orientation, homologous recombination across these sequences would lead to excision of the intervening DNA. pED991 may possibly be a product of such an excision. If so the loss of Tc^R in pED991 would be analogous to the loss of Cm^R mediated by homologous recombination across the flanking direct repeats of IS1 in $\lambda::Tn9$ phages (MacHattie and Jackowski, 1977), and to the excision of resistance determinant regions bounded by copies of IS1, from several IncFII R factors in Salmonella typhimurium and Proteus mirabilis (Watanabe et al, 1964b; see Hu et al, 1975). In contrast, the analogous recombination event between the indirectly repeated ISN elements on R46 causes inversion of the intervening segment, and is responsible for the difference between R46A and R46B (Ch. 4.c.iv).

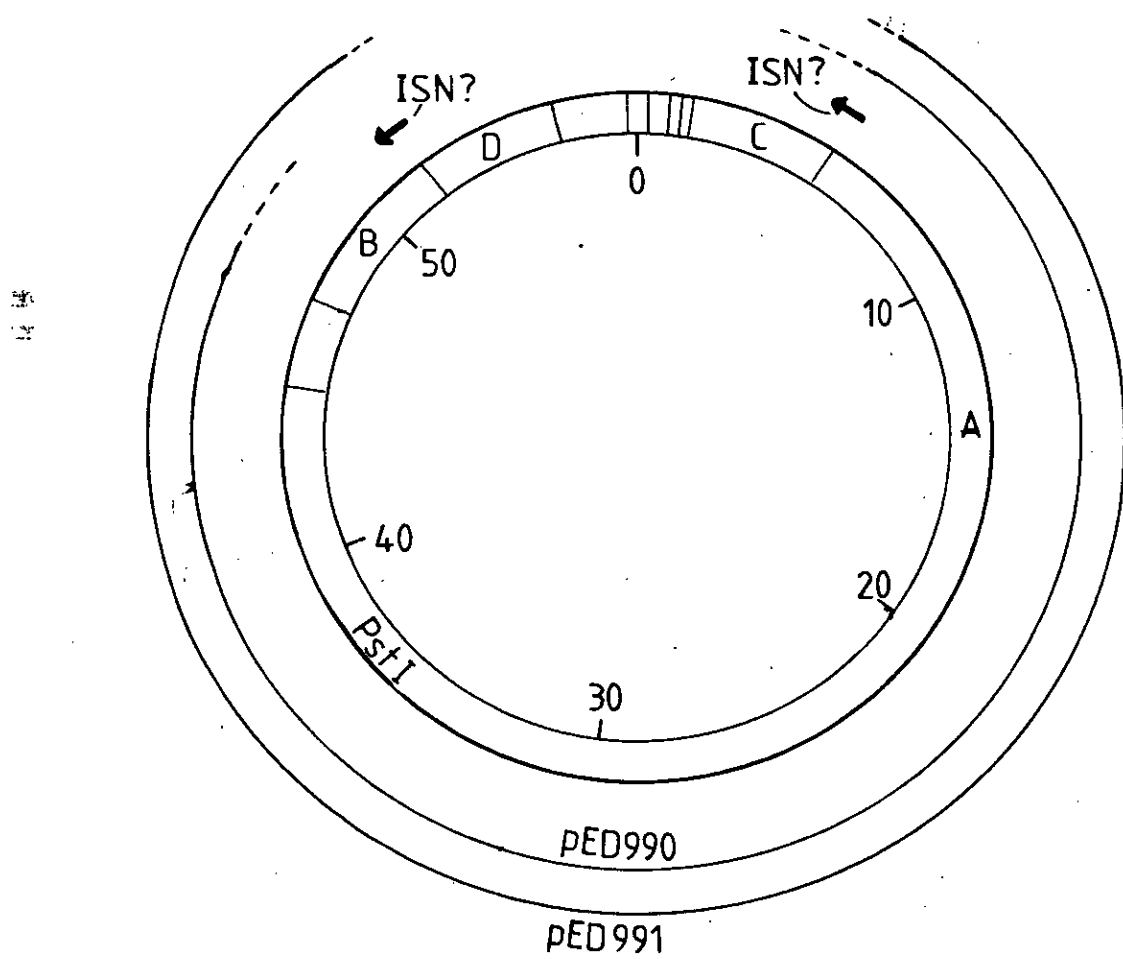
Fig. 7.4

Simplified map of N3 showing the DNA contained in the spontaneous deletion derivatives pED990 and pED991. The approximate end-points of the deletions are shown in relation to the relevant PstI fragments of N3 and to the possible locations of two directly repeated copies of the ISN element.

pED990 may have been formed by an ISN-mediated deletion, and pED991 by recombinational excision of the segment between two ISN repeats.

Lettering of PstI fragments and map co-ordinates are as in Fig. 4.6.

Fig. 7.4



In addition to the ISN elements there is suggestive evidence for a number of other transposable elements on R46 and N3. Firstly if both copies of ISN on each plasmid are indeed transposable this raises the interesting possibility that the intervening DNA segments are transposable also, by virtue of the flanking insertion sequences.

In R46 this segment determines resistance to arsenate, arsenite and antimony (III), and to tetracycline, while the relevant region of N3 contains the Tc^R determinant of that plasmid. No evidence of the transposition of either of these regions has yet been obtained, however. Transposition of Asa^R , Asi^R and Ant^R has not been reported in other plasmids, although the equivalent resistance region on plasmids of Staphylococcus aureus is also bounded by inverted repeats (Novick et al, 1979b), and transposable resistance to other metal ions is known (Bennett et al, 1978). Yamamoto et al (1981a) have briefly referred to the existence of an Ap^R transposon on R46, and tentative evidence of such an element was described above. If this data is confirmed, the transposon is approximately 3 kb long, and must differ substantially from the 19.6 kb element Tn2603, the only known example of a transposon that encodes a β -lactamase of the oxacillin-hydrolysing variety (Yamamoto et al, 1981a, 1981b). The presence of inverted repeats of approximately 350 bp flanking the Uvp determinant and/replication region of R46 possibly the (Langer et al, 1981; Ch. 4.c.iii; Fig. 6.5) raises the intriguing possibility that this region might be transposable, although no evidence of this has been obtained. To detect such transposition, if it were to occur at low frequency, it may be necessary to use R46 derivatives lacking all other transposable segments. Finally, the N3/pED936 heterouplex molecule shown in Fig. 6.9 revealed a

stem-loop structure of approximately 1.45 kb in total size, flanked by inverted repeats of approximately 200 bp (see Ch. 6.c.iv). Again this structure is suggestive of a transposable element, although none of the well characterised elements of similar length have inverted repeats as long as 200 bp. It is possible that this length is an overestimate, however, since the lengths of inverted repeats in other transposable elements, estimated by electron microscopy as 100-200 bp, have subsequently been shown by DNA sequence analysis to be considerably shorter, for example the 38 bp terminal repeats of Tn3 (Heffron et al, 1975; Cohen et al, 1979). The map position of the 1.45 kb stem-loop structure on N3 can not yet be determined unambiguously.

In conclusion, the resistance determinant regions of R46 and N3 contain a variety of structures which may represent transposable elements, although direct evidence of transposition has not been obtained in many cases. However, at least one of the ISN elements on R46 is an active insertion sequence, and N3 may contain two similar elements at least one of which is also transposable.

CHAPTER 8. THE ORIGIN OF TRANSFER OF R46

(a) Introduction

Conjugative transfer of plasmid DNA has been shown to be unidirectional for members of incompatibility groups FI and P (Jacob and Wollman, 1961; Rupp and Ihler, 1968; Ohki and Tomizawa, 1968; Al-doori et al, 1981), and transfer is believed to be initiated from a fixed origin on the molecule. These are probably general properties applicable to other conjugative plasmids.

Willetts (1972) and Guyer and Clark (1976) identified a region of the plasmid F, deletions of which^{gave} rise to a cis-dominant transfer defect. They suggested that this region includes the origin of transfer, and the cis-acting sequence has been designated oriT. Willetts (1972) and Reeves and Willetts (1974) have proposed that F DNA transfer is initiated at a site-specific nick introduced at oriT by a plasmid-coded endonuclease, and Everett and Willetts (1980) have recently provided evidence for such nicking. Although initially identified solely as a cis-acting region essential for plasmid transfer, oriT has also been defined as the nucleotide sequence recognised by the nicking endonuclease, and within which the nick is introduced (Willetts, 1972). As expected, recombinant plasmids containing the oriT region of F cloned in non-transmissible plasmid vectors are mobilised at high frequencies by Flac (Achtman et al, 1978; Thompson and Achtman, 1978).

A cis-acting region necessary for transfer has also been identified on the IncP plasmid RK2, and a Tra⁻ recombinant plasmid containing this segment of the molecule was efficiently mobilised by a Tra⁺ RK2 derivative (Guiney and Helinski, 1979; Thomas et al, 1979). This region is therefore presumed to contain oriT of RK2.

The small non-conjugative plasmids ColE1 and CloDF13 have each been shown to contain a cis-acting region required for their mobilisation by particular conjugative plasmids present in the same cell (Warren et al, 1978; van de Pol et al, 1978). These regions have been designated bom (basis of mobility). Although the genetic properties of bom are formally analogous to those of oriT in F, there is as yet no evidence that ColE1 or CloDF13 are transferred in a unidirectional linear manner from a fixed origin (see Clark and Warren, 1979).

Many plasmids can be isolated from their host cells in the form of supercoiled DNA-protein 'relaxation complexes', which upon treatment with SDS, Ethidium Bromide, or proteases, are converted to nicked open circular forms (Clewell and Helinski, 1969; Kline and Helinski, 1971; Kuperstoch-Portnoy et al, 1974; Guiney and Helinski, 1979). The relaxation of RK2, ColE1 and RSF1010 occurs by the introduction of a site-specific nick, and these nick sites lie within regions of the plasmids containing oriT or bom (Guiney and Helinski, 1979; Thomas et al, 1979; Tomizawa et al, 1977; Warren et al, 1978; Nordheim et al, 1980; K. Derbyshire and N.S. Willetts, unpublished results). This suggests that relaxation complexes are involved in conjugal transfer of plasmid DNA, and that in vitro nicking of relaxation complexes may mimic in vivo nicking at oriT. The proposed relationship between relaxation complexes and the initiation of plasmid transfer may not be obligatory, however, since attempts to isolate relaxation complex of the mobilisable plasmid CloDF13 have been unsuccessful (J. Nijkamp, pers. comm.), and Johnson et al (1981) have recently reported that the relaxation observed in F DNA-protein complexes is not site-specific.

Alternatively, site-specific relaxation complexes of these plasmids may exist in vivo, but cannot be isolated by the methods so far applied. Preliminary experiments were conducted to determine whether R46 can be isolated as a DNA-protein complex that relaxes at a specific site, so that the site of any such nick could be localised. The results of these experiments were inconclusive, however.

The IncN plasmid R46 determines a conjugative transfer system that is distinct from those previously studied, and results presented here identify a cis-acting region of R46 essential for transfer which presumably includes oriT. This region has been localised in relation to the plasmid transfer genes by complementation experiments using deletion and recombinant derivatives of R46, and the cis-acting sequence has been cloned using a selection procedure for mobilisable recombinant plasmids, so that it may be studied in more detail.

(b) Genetic Mapping of oriT

The recombinant plasmids pED935 and pED936 are self-transmissible and contain a 22 kb segment of R46 DNA cloned into pBR325 (Ch. 5.b.v). pBR325 itself is not mobilised by R46, indicating that the 22 kb cloned segment in pED935 and pED936 includes a cis-acting DNA sequence as well as all the R46 transfer genes. This sequence is most probably the origin of transfer of R46 and will be referred to as oriT.

In order to locate the cis-acting sequence within the 22 kb region of R46, a number of Tra⁻ recombinant and deletion plasmids carrying defined segments of this region were tested for their

Table 8.1 Frequencies of mobilisation of Tra⁻ plasmids by Tra⁺ plasmids.

<u>Tra⁻ plasmid</u>	<u>Tra⁺ plasmid</u>			
	<u>pED912</u>	<u>pED935</u>	<u>R46</u>	<u>N3</u>
pED923		1.0		
pED927	<5x10 ⁻⁴			
pED928	3.0			
pED930	<5x10 ⁻⁴			
pED933			1.2x10 ⁻⁵	
pED938	1.2			2.7
pED939	2.7			
pED940		1.5		
pED941		2.5x10 ⁻⁷		
pED967	2.3x10 ⁻³			
pBR325	<10 ⁻⁴		4x10 ⁻⁷	
pBR327	<10 ⁻⁴			
pED825	<10 ⁻⁴			<10 ⁻⁴

Frequencies of mobilisation of Tra⁻ plasmids are calculated in relation to the transfer frequency of the Tra⁺ plasmid present in the same JC6310 host strain. The recipient was ED3818. Data are from 30 min membrane filter matings.

ability to be mobilised by Tra^+ R46 derivatives in a recA^- host. Tra^- plasmids containing oriT of R46 were expected to be complemented for transfer and therefore mobilised at high frequency, whilst those deleted for the sequence would have a cis-dominant transfer defect.

To ensure that the Tra^+ and Tra^- R46 derivatives would co-exist stably in the donor cells, pairs of compatible plasmids were chosen for these experiments. R46 deletion derivatives that express IncN incompatibility were therefore tested for mobilisation by pED935 (with pBR325 incompatibility), while recombinant plasmids consisting of R46 restriction fragments cloned in pBR325 were tested in the presence of the IncN Tra^+ plasmid pED912 (Table 5.1). Stable double plasmid strains were constructed in the RecA^- strain JC6310 and used as donors in membrane filter experiments (Ch. 2.c.ii) with the Nal^R recipient strain ED3818. Transconjugants bearing markers characteristic of each plasmid were selected, and mobilisation frequencies of the Tra^- plasmids were calculated relative to the transfer frequency of the Tra^+ plasmid in the same donor strain (see Table 8.1).

The transfer region of R46 is contained within three BglII fragments, BglII-F, G and A, and these are cloned in plasmids pED928, 930 and 927, respectively (see Ch. 5.b.iii, Fig. 6.7). The results in Table 8.1 show that of these three plasmids only pED928 was mobilised at high frequency by pED912, indicating that oriT lies within BglII-F of R46 (co-ordinates 12.95 to 17.45 kb, Fig. 6.7). The deletion plasmids pED940 and pED941 contain R46 DNA from approximately co-ordinate 50kb clockwise to approximately co-ordinates 19.5 kb and 14.5 kb respectively (Table 5.2; Fig. 6.7). pED940

therefore includes all of BglIII-F, while pED941 is deleted for part of this fragment, and Table 8.1 shows that pED940 was mobilised at high frequency whereas pED941 was not. pED941 therefore has a cis-dominant transfer defect, indicating that oriT of R46 is located between approximately co-ordinates 14.5 kb and 17.45 kb within BglIII-F (Fig. 6.7). As expected, the Tra⁻ plasmids pED923 and pED974 containing this region were mobilised at high frequency (Table 8.1).

(c) (i) Cloning of oriT: construction and properties of pED938

In order to confirm the location of oriT within the 4.6 kb BglIII-F fragment, and to identify its position more precisely, oriT was cloned on a smaller restriction fragment. This was achieved by cloning HaeII restriction fragments of R46 DNA into the vector pED825, and selecting recombinant plasmids that could be mobilised by the Tra⁺ R46 derivative pED912. HaeII recognises and cleaves the partially degenerate hexanucleotide sequence PuGCGC↓Py, and the largest HaeII restriction fragment of R46 DNA is smaller than 1.8 kb.

The multicopy Ap^R plasmid pED825 (Fig. 8.1) is composed of HaeII fragments A and E of pBR322 (R. Everett and N.S. Willetts, unpublished; Sutcliffe, 1978). One of the two HaeII sites in pED825 lies within the essential replication region so in order to reconstitute viable plasmids following HaeII cleavage, the two HaeII fragments must be precisely re-ligated at this site. The other HaeII site is available for the insertion of DNA and pED825 may therefore be used as a cloning vehicle for HaeII restriction fragments.

R46 and pED825 DNA was digested with HaeII, mixed, and ligated. The recA⁻ strain ED8641 (pED912) was transformed with this mixture,

and Ap^R transformants were selected in broth. The Ap^R transformants were then used as donors in a 30 minute membrane filter mating with the Nal^R recipient strain ED3818, selecting Ap^R[Nal^R] transconjugants. Such transconjugants should arise only if pED825 recombinant derivatives containing oriT or R46 were mobilised by pED912 and hence transferred into ED3818. The Ap^R[Nal^R] transconjugants were screened for sensitivity to tetracycline to identify those which had not co-inherited pED912, and DNA from 22 such Ap^R Tc^R isolates was examined by agarose gel electrophoresis to determine the sizes of the plasmids they contained. Purified DNA of 4 of the smallest plasmids was digested with HaeII, and the restriction cleavage fragments examined by gel electrophoresis. (Fig.8.3) One of the plasmids numbered pED938, consists of the two HaeII fragments of pED825, plus a single additional 1.4kb HaeII fragment. (Figs.8.2,8.3)

The frequency of mobilisation of pED938 from the recA⁻ strain JC6310 (pED912, pED938) was measured in quantitative membrane filter crosses with ED3818. pED938 was mobilised at approximately 1.2 times the transfer frequency of pED912 (Table.8.1) Since the pED825 vector plasmid is mobilised by pED912 at frequencies less than 10⁻⁴ (Table 8.1) this confirms that the 1.4 kb HaeII fragment cloned in pED938 contains the origin of transfer of R46. Comparison of the HaeII restriction fragments of R46, pED938, pED928 and pBR325 DNA in agarose gel electrophoresis showed that a single fragment of this size is present in R46 and pED928, but not in pBR325. Since pED928 consists of the R46 BglIIF fragment cloned in pBR325, this supports the conclusion reached in section 8.b. that oriT of R46 lies within BglII fragment F.

Fig. 8.1 Map of the HaeII cloning vector pED825.

One of the two HaeII sites lies within the region essential for replication; the other is available for insertion of DNA fragments.

Fig. 8.2 Map of pED938.

pED938 is composed of a 1.4 kb HaeII fragment cloned in the vector pED815 (see Fig. 8.1). The cloned fragment is indicated by the thicker line. pED938 contains 4 TaqI sites, of which one lies within the replication region and another within the Ap^R determinant. To reconstitute viable Ap^R plasmids following TaqI cleavage, three of the TaqI fragments must be re-ligated in their original order and orientation, but the fourth fragment is non-essential. This fragment was deleted in the formation of pED939, and is indicated in the figure.

Fig. 8.1

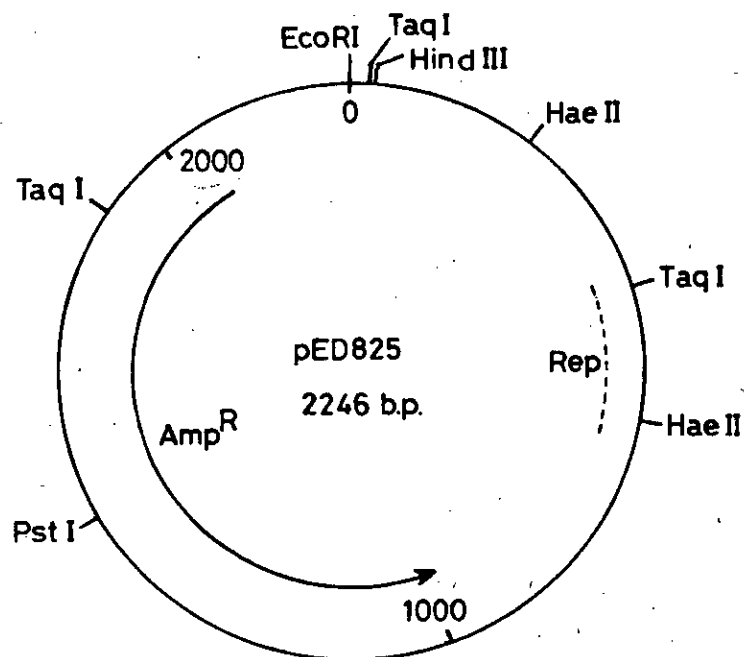
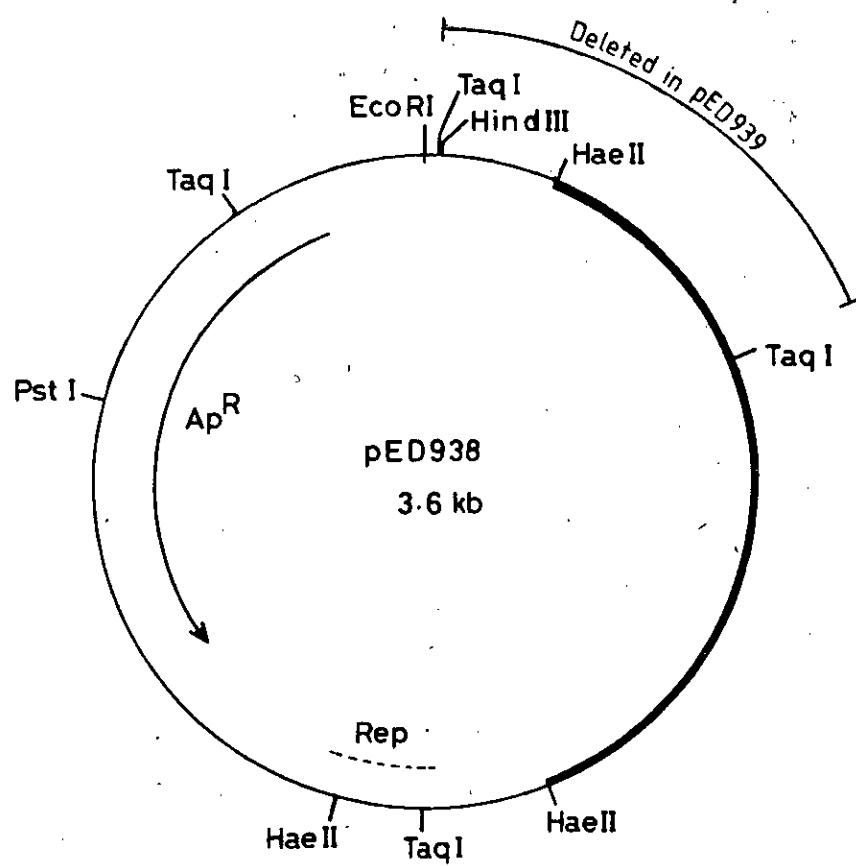


Fig. 8.2



In a parallel experiment pED928 DNA was digested with HaeII, ligated, and used to transform ED8641 (pED912) with selection for mobilisable Ap^R transformant plasmids as described above. In this case deletion derivatives of pED928 should have been recovered composed of the two HaeII fragments of pBR325 necessary for plasmid replication and Ap^R, plus a sub-fragment from BglIII-F containing oriT. The two smallest mobilisable plasmids recovered from this experiment, one of which was pED995, both contained two or more HaeII fragments in addition to those required for plasmid replication and Ap^R. The only extra HaeII fragment common to both plasmids, however, co-migrated with the cloned fragment from pED938 in gel electrophoresis. This result further confirms that oriT or R46 lies on the 1.4 kb HaeII fragment within BglIII-F.

Langer and Walker (1981) have mapped two cleavage sites for the restriction endonuclease HpaI within the BglIII fragment of pKM101 that corresponds to BglIII-F of R46. These sites are symmetrically placed 0.9 kb from the BglIII sites, and lie 2.8 kb apart. The 1.4 kb HaeII fragment of pED938 containing oriT contains no cleavage sites for either BglIII or HpaI, so must therefore lie within the 2.8 kb HpaI fragment in BglIII-F. (see Fig. 8.4) In conjunction with the genetic evidence presented in section 8.b. These data indicate that oriT of R46 is located between co-ordinates approximately 14.5 and 16.5 kb. It therefore lies at or close to the anticlockwise end of the R46 transfer region (Fig.6.6).

An unusual property of the cloned HaeII restriction fragment in pED938 is that its rate of migration in polyacrylamide gel electrophoresis, relative to standard fragments of known size, is significantly

Fig. 8.3 HaeII restriction analysis of pED938, showing differential migration of the cloned HaeII fragment in agarose and polyacrylamide gel electrophoresis.

(a) 1.2% agarose gel electrophoresis

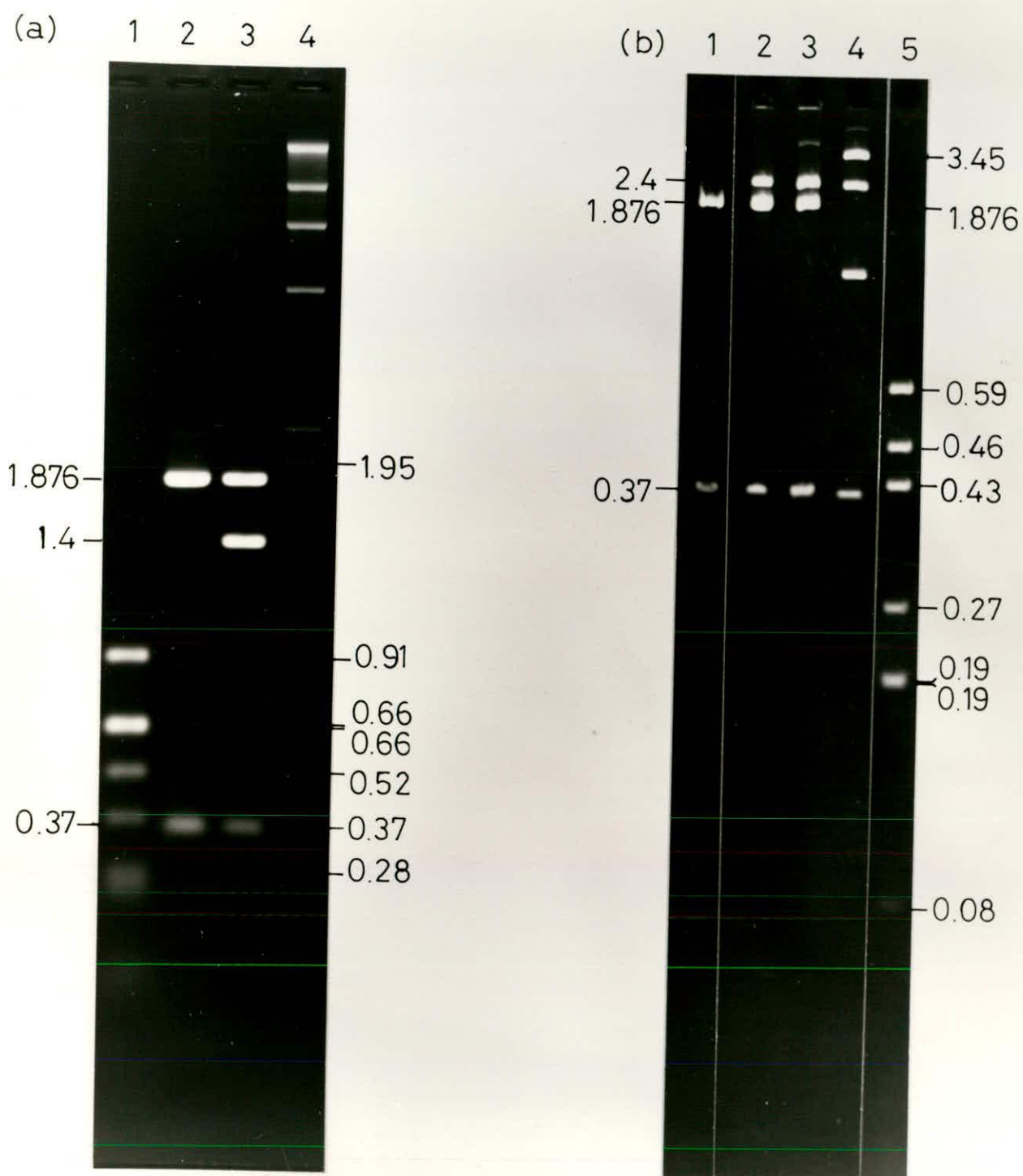
<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pBR322	<u>AluI</u>
2	pED825	<u>HaeII</u>
3	pED938	<u>HaeII</u>
4	λ 7	<u>HindIII</u>

(b) 5% polyacrylamide gel electrophoresis

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pED825	<u>HaeII</u>
2	dimeric isolate similar to pED938	<u>HaeII</u>
3	pED938	<u>HaeII</u>
4	pED995	<u>HaeII</u>
5	pED825	<u>HaeIII</u>

- Notes: i) Sizes of fragments marked in kilobases are derived from nucleotide sequence data (Sutcliffe, 1978), with the exception of the 1.4 kb and 2.4 kb values, which are estimated from these gels.
- ii) Compare tracks (a) 2 and 3 with tracks (b) 1 and 3. The cloned HaeII fragment in pED938 migrates below the 1.87 kb vector fragment in agarose but above it in acrylamide, leading to size estimates of 1.4 kb and 2.4 kb, respectively. pED995 contains the same fragment cloned in a different vector.

Fig. 8.3



slower than its corresponding rate in agarose gels. pED938 contains the two HaeII fragments of the vector plasmid pED825, in addition to the cloned fragment. These are both of known nucleotide sequence and are 1876 bp and 370 bp in length (Sutcliffe, 1979). In electrophoresis in 1.2% agarose gels the cloned HaeII fragment containing oriT migrated between the two vector fragments (Fig. 8.3), and its size was calculated as 1.4 kb. When the HaeII restriction fragments of pED938 DNA were separated by electrophoresis in a 5% polyacrylamide (5% glycerol) gel, however, the cloned fragment migrated more slowly than either of the two vector fragments, and its size was estimated to be approximately 2.4 kb (see Fig. 8.3). In another 5% polyacrylamide gel (not containing glycerol), electrophoresis was carried out at a higher voltage gradient, causing the gel apparatus to feel warm to the touch. Under these conditions the cloned HaeII fragment of pED938 co-migrated with the 1876 bp fragment. In other cases in which restriction fragments have altered mobilities in different gel systems (see below) fragments of known size appear to be retarded in acrylamide gels relative to size standards, rather than accelerated in agarose. Thus the value of 1.4 kb for the cloned HaeII fragment in pED938, derived from agarose gel electrophoresis, was assumed to be the best estimate of the fragment's actual size. The apparent retardation of this fragment in polyacrylamide gels is perhaps reduced at higher temperatures, although further investigations of this effect are required before firm conclusions can be drawn.

(c) (ii) Restriction mapping of pED938

In order to allow oriT to be located more precisely within the 1.4 kb HaeII fragment in pED938, it was first necessary to construct

a map of restriction sites within this fragment. The fragment contained no cleavage sites for any of the fourteen hexanucleotide specific-restriction enzymes EcoRI, HindIII, BamHI, BglII, PstI, SalI, KpnI, SmaI, XhoI, AvaI, AccI, HpaI, PvuII, and SacI, and no site for the heptonucleotide-specific enzyme BstEII (Fig. 4.2; unpublished data; Langer and Walker, 1981). A restriction map was therefore constructed using enzymes which recognise tetranucleotide sequences and hence cleave DNA more frequently than hexanucleotide-specific enzymes.

pED938 DNA was digested with each of the tetranucleotide-specific restriction endonucleases TaqI, MspI, Sau3A, AluI, and HaeIII, either singly or in pairs. The sizes of the resulting cleavage fragments were then estimated from their mobilities in gel electrophoresis in order to construct a restriction map by methods similar to those described in Chapter 4. Because of the relatively small size of cleavage fragments generated by these enzymes it was usually necessary to conduct electrophoresis in polyacrylamide gels to resolve the DNA fragments (e.g. see Fig. 8.5). Since the cloned HaeII fragment in pED938 is disproportionately retarded in polyacrylamide gels in relation to its probable size, estimates of the sizes of some fragments containing this DNA may therefore have been subject to substantial errors. The positions of the restriction cleavage sites shown in Fig. 8.4 within the cloned segment of pED938 are thus only approximate.

(c) (iii) Construction of pED939 by deletion from pED938

Fig. 8.4 shows that the cloned HaeII fragment in pED938 contains a single cleavage site for the restriction endonuclease TaqI. In order to determine the location of oriT in relation to this site, one of the two pED938 TaqI fragments containing R46 DNA was deleted in vitro and the resulting plasmid tested for the presence of oriT. TaqI recognises

and cleaves the nucleotide sequence T⁺CGA leaving dinucleotide 5' terminal extensions suitable for ligation. There are three other TaqI cleavage sites within pED938 (Fig. 8.2). One lies in the plasmid replication region, another within the Ap^R determinant, and the third in a non-essential region between the EcoRI and HindIII cleavage sites. Following TaqI cleavage and ligation of pED938 DNA, three of the four Taq I fragments must be re-ligated in their original order and orientation to allow the recovery of viable Ap^R plasmids. The fourth TaqI fragment, which is approximately 700 base pairs in size, can therefore be deleted. This fragment contains approximately 500 base pairs of cloned R46 DNA.

pED938 DNA was digested with TaqI for 1 hour at 65°C and then extracted with phenol to remove the enzyme. Following ether extraction and ethanol precipitation, the DNA fragments were ligated and used to transform ED8654. Ap^R transformants were selected and screened for the presence of plasmids smaller than pED938 by gel electrophoresis of small scale cleared lysate preparations. TaqI restriction analysis of one such plasmid, numbered pED939, confirmed that it has lost the 700 bp fragment (Fig. 8.6).

pED939 was tested for mobilisation by pED912 from JC6310 (pED912, pED939) using membrane filter matings. As shown in Table 8.1 the plasmid was mobilised at 2.7 times the transfer frequency of pED912, indicating that pED939 retains all of the R46 oriT sequence. This localises oriT within the 900 bp of R46 DNA present in pED939 (Fig.8.4).

(c) (iv) Sub-cloning from pED938

A number of attempts were made to sub-clone Sau3A restriction fragments from pED938 with selection for mobilised recombinants, in

Fig. 8.4

Partial map of pED938 showing the approximate positions of tetranucleotide-specific restriction enzyme sites within the cloned HaeII fragment (indicated by the thicker line). This fragment contains oriT and maps within BglII fragment F of R46, which is also shown. The R46 DNA contained in the oriT⁺ pED938 deletion derivative pED939 is indicated below the map, as is the approximately 215 bp Sau3A fragment contained in the poorly mobilisable sub-clone pED967.

The positions of restriction sites in the vector DNA were obtained from nucleotide sequence data (Sutcliffe, 1978).

Fig. 8.4

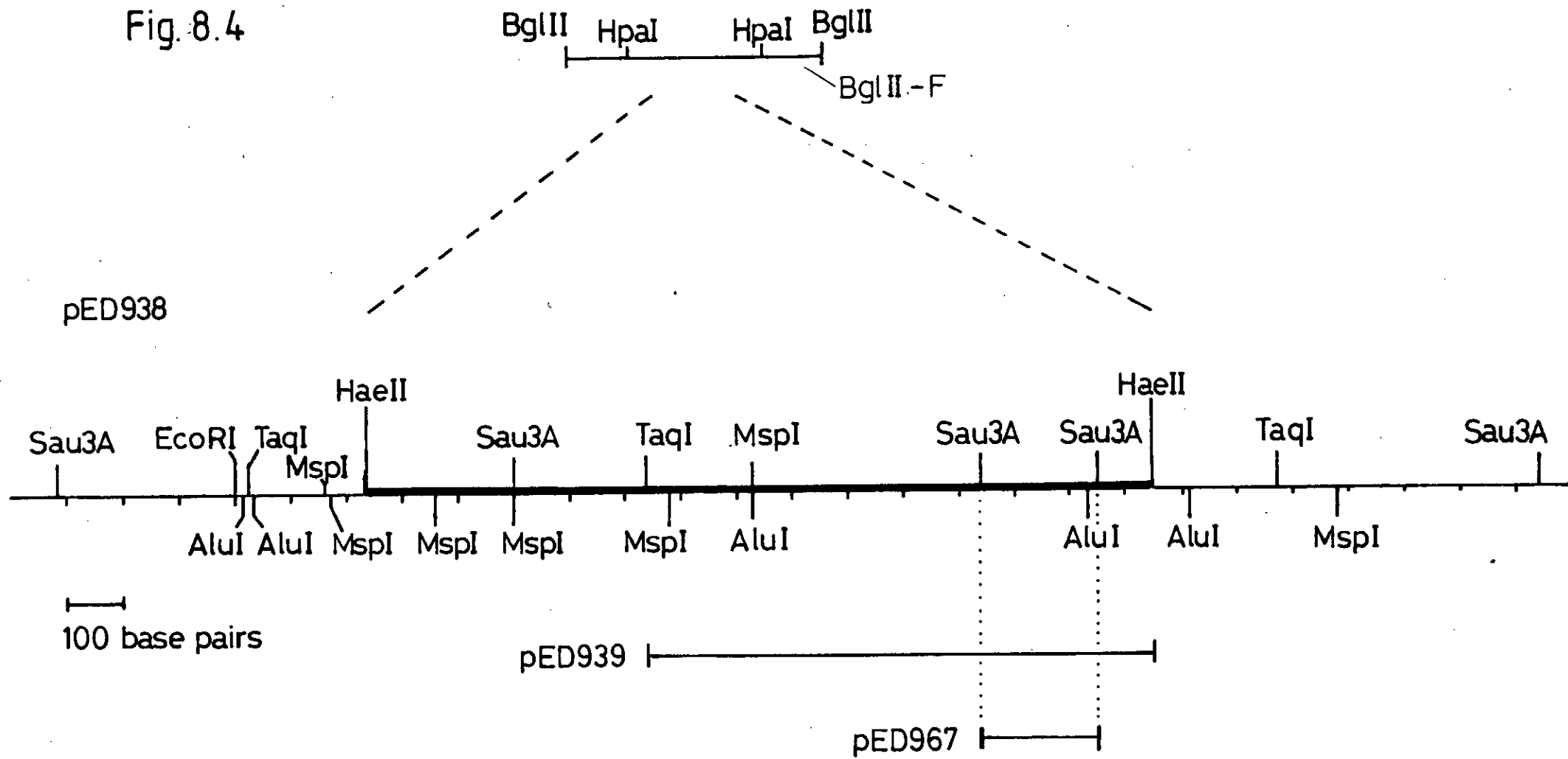


Fig. 8.5. 5% polyacrylamide gel electrophoresis: examples of restriction enzyme digestion data used in construction of the restriction map of pED938 (Fig. 8.4).

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease(s)</u>
1	pED938	<u>Sau3A</u>
2	pED938	<u>Sau3A</u> + <u>AluI</u>
3	pED938	<u>AluI</u>
4	pBR322	<u>AluI</u>

Sizes of AluI fragments of pBR322 are marked in base pairs.

Fig. 8.6 1.2% agarose gel electrophoresis: TaqI restriction analysis of pED939

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pED825	<u>TaqI</u>
2	pED939	<u>TaqI</u>
3	pED938	<u>TaqI</u>

Sizes of the TaqI fragments of pED825 are derived from nucleotide sequence data (Sutcliffe, 1978), and those of the non-vector fragments of pED938 are estimated from data not shown. Note that the 700 bp fragment of pED938 is missing in pED939.

Fig. 8.7. 5% polyacrylamide gel electrophoresis: Sau3A restriction analysis of pED967.

<u>Track</u>	<u>DNA</u>	<u>Restriction endonuclease</u>
1	pED967	<u>Sau3A</u>
2	pBR327	<u>Sau3A</u>
3	pBR322	<u>AluI</u>

Sizes of AluI fragments of pBR322 are marked in base pairs.

Comparison of tracks 1 and 2 shows that pED967 contains a Sau3A fragment of approximately 215 bp cloned in pBR327.

Fig. 8.5

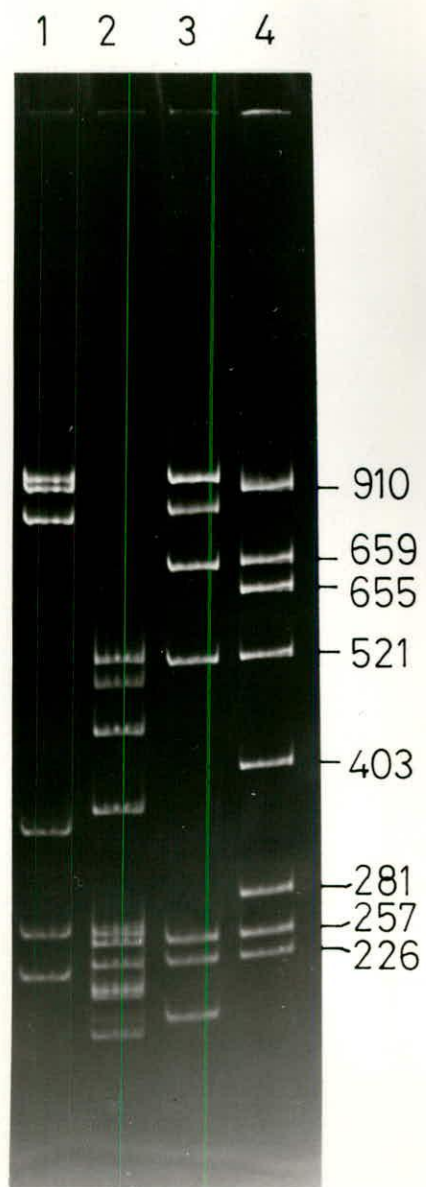
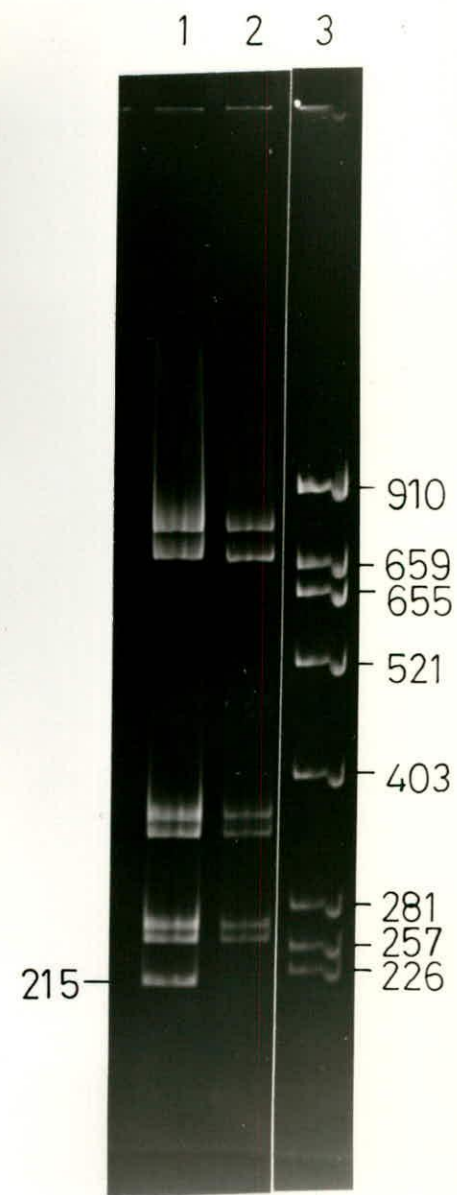


Fig. 8.6



Fig. 8.7



the hope that this would locate oriT more accurately and perhaps facilitate DNA sequencing of the relevant region.

The restriction endonuclease Sau3A produces 5'-GATC single strand extensions complementary to those produced by BamHI, allowing Sau3A fragments of pED938 to be cloned into the unique BamHI sites of the pBR322-related vector plasmids pAT153, pBR327, or pBR328. The recombinant plasmids were introduced into ED8641 (pED912) by transformation, selected for the ability to be mobilised, and screened to identify those of smallest size, by methods described in section 8.(c)(i).

In two separate experiments the smallest recombinant plasmids recovered contained 1.9 kb of inserted DNA; this size corresponds to all three Sau3A fragments comprising the R46 DNA in pED938. In a third experiment (using pBR327 as vector) a plasmid, pED967, containing only one of these fragments, was obtained. The cloned Sau3A fragment in pED967 is only 215 bp in size (Fig. 8.7). Although it was originally selected as a recombinant plasmid mobilised by pED912, pED967 was only transferred with a frequency of 3×10^{-3} relative to pED912 when tested in quantitative crosses (Table 8.1). This suggests that pED967 may only contain a part of the oriT sequence required for high frequency mobilisation.

(d) Discussion

cis-acting DNA sequences required for the transfer of conjugative plasmids have previously been mapped on F and RK2 in relation to the transfer genes of these plasmids. oriT of F lies at one end of the single large transfer region (Willetts, 1972; Thompson and Achtman 1978). In RK2 the relaxation complex nick site and presumptive origin of transfer lies at or close to one end of the TraI region (Guiney and

Helinski, 1979; Barth 1979)). In both cases the direction of plasmid transfer from these origins is such that the adjacent transfer genes are transferred last (Willetts, 1972; Al-doori et al, 1981). Results presented here establish that oriT or R46 is also located at or close to one end of the plasmid transfer region, though the direction of DNA transfer has not yet been determined. It is possible that the transfer genes of R46 are organised as a large operon as in F (see Willetts and Skurray, 1980), and that the position of oriT at one end of these transfer regions may be a consequence of this operon structure, since protein-DNA interactions at an oriT site within an operon might interfere with its transcription.

The low incidence of hexanucleotide-specific restriction cleavage sites throughout the R46 transfer region (Ch.6. e) is exemplified by the 1.4 kb of R46 DNA cloned in pED938, which includes oriT. This segment contains no cleavage sites for any of 14 hexanucleotide-specific enzymes, and contains fewer sites for the tetranucleotide-specific enzymes TaqI, Sau3A, and AluI than expected in a random DNA sequence of the same length. The cloned DNA fragment in pED938 also exhibited the unusual property that in polyacrylamide gel electrophoresis its rate of migration relative to fragments of known size was considerably slower than in agarose gels, such that estimates of the size of the fragment based on its mobility in the two gel systems differ by up to 1000 bp.

The rate of migration of DNA fragments in polyacrylamide gel electrophoresis may be affected by their nucleotide composition and sequence as well as their molecular weight, but aberrant migration cannot always be ascribed to the overall AT/GC composition ratio of a fragment. The HpaII fragment C3 of bacteriophage T7 DNA for example, is retarded in polyacrylamide gels relative to other fragments but has a

nucleotide composition of 53.6% AT (J. Boothroyd, 1979; Dunn and Studier, 1981). Extreme retardation on polyacrylamide gels has also been observed for DNA fragments from the attP and ori regions of phage λ (W. Ross, personal communication). Both these regions are involved in protein-DNA interactions and Hobom *et al* (1979) have proposed that the nucleotide sequence at λ ori will form a 'clover-leaf' secondary structure. Since the origin of transfer of R46 is also a site of protein-DNA interaction, it may also form a characteristic secondary structure *in vivo* to allow protein recognition. Similar secondary structures formed during polyacrylamide gel electrophoresis, but not in agarose, might account for the differential relative migration of the oriT⁺ HaeII restriction fragment in pED938. A relative retardation in polyacrylamide gels was also observed for certain sub-fragments of this DNA, but the available data are insufficient to indicate whether the phenomenon can be ascribed specifically to DNA fragments containing oriT.

Amongst IncF plasmids there are at least three different oriT sequence specificities, so that, for example, neither R1-19 nor R100-1 will recognise oriT of F (Everett and Willetts, 1980). In order to investigate whether a similar difference in specificity exists between R46 and N3, pED938, a clone containing oriT or R46, was tested for mobilisation by N3. Table 8.1 shows that pED938 was transferred from JC6310 (N3, pED938) at over twice the frequency of N3 itself. Although this suggests that N3 efficiently recognises oriT or R46, it is also possible that pED938 includes an R46 gene involved in recognition of this origin. Plasmids containing R46 oriT in shorter DNA segments than that cloned in pED938, should therefore be tested in this manner before firm conclusions can be drawn.

Table 8.2 shows that while pED939 was mobilised at high frequency by pED912, pED967 was mobilised approximately 1000-fold less efficiently than pED939, but significantly better than pBR327. A simple interpretation of these data is that while pED939 includes all of oriT, pED967 contains only part of the relevant sequence. The oriT sequence would thus include one of the two Sau3A sites flanking the 215 bp fragment cloned in pED967.

Alternatively, the sequence of oriT may be longer than 215 bp and include both the Sau3A sites flanking the cloned fragment in pED967. This would be plausible if extensive secondary structure in the region were required for normal recognition of oriT by the proteins which initiate DNA transfer. It might also account for the frequent recovery of mobilised recombinant plasmids containing three cloned Sau3A fragments, although this may have been fortuitous.

For plasmids to be transferred by conjugation, they must be recognised specifically by the transfer gene products at the conjugation bridge between cells. This is clearly implied by the mobilisation, by certain conjugative plasmids only, of non-conjugative plasmids such as ColE1 which encode their own conjugal DNA metabolism (Mob) functions. It has been assumed above that the cis-acting region of R46 under investigation corresponds to a single cis-acting DNA sequence required for the initiation of plasmid transfer. The possibility remains, however, that this region may contain two cis-acting sequences, one recognised by a site-specific endonuclease (oriT), and another sequence, distinct from this, which is essential for physical transfer of the plasmid. Such a sequence might be required to interact with the conjugation bridge between cells, to provide specificity of recognition

between the plasmid DNA and the physical apparatus of conjugation.

DNA sequencing of the oriT region of R46, together with the construction of further deletions and other derivatives, should permit more extensive characterisation of the cis-acting sequence(s) required for plasmid transfer.

CHAPTER 9: FINAL DISCUSSION

The organisation of genetic material in conjugative antibiotic resistance plasmids has previously been studied mainly for members of the incompatibility groups IncFII, IncP, and IncW (Sharp et al, 1973; Tanaka et al, 1976; Blohm and Goebel, 1978; Timmis et al, 1978; Alton and Vapnek, 1978; Ward and Grinsted, 1978; Gorai et al 1979; see Thomas, 1981) and this thesis provides similar data for two representative IncN plasmids. Although the number of incompatibility groups represented in the above studies is small compared with the total number of such groups and their apparent diversity (see Chapter 1), several general observations can be drawn concerning the genetic organisation of these plasmids.

Determinants of functions essential for plasmid maintenance and replication are often found clustered in a single region that includes the major origin of replication (See Ch. 6.d), although in some cases there may be alternative origins elsewhere on the molecule (e.g. see Timmis et al, 1981). Similarly, the conjugal transfer genes are also usually clustered together in a relatively large contiguous region adjacent to oriT (see Ch. 6.d, Ch. 8), and in some cases these genes are organised as an operon. Replication and transfer are the two fundamental and essential properties of conjugative plasmids, and as a consequence of the organisation of their determinants in blocks of contiguous DNA, the determinants of non-essential functions such as antibiotic resistance are often found in a separate region of the molecule.

The evolutionary significance of the features of plasmid organisation is entirely a matter for speculation, but the available data allow some plausible suggestions. The extensive homology exhibited

between the transfer regions of plasmids in the same incompatibility group, such as R46 and N3 (Ch.6. (c) (iv), and see Ch. 1), and in some cases between plasmids in different groups (Sharp et al 1973, Falkow et al, 1974), indicates that these regions are highly conserved during plasmid evolution. The same may be true of replication regions. Antibiotic resistance genes, however, often differ in their nature and position in closely related plasmids (e.g. compare Figs. 6.6 and 6.10), suggesting that plasmids may evolve by the addition of such genes to an ancestral replication and transfer 'unit', from which they may also subsequently be deleted. The asymmetric distribution of restriction endonuclease cleavage sites in IncN and other conjugative antibiotic resistance plasmids may reflect this mode of evolution: the scarcity of such sites in the replication and transfer regions may be the result of selection over a long period of time and in a wide variety of hosts, while the greater number of sites in the vicinity of antibiotic resistance determinants may reflect their relatively recent acquisition (see Chapter 6.d). Transposition is a likely mechanism for such acquisition.

Given the clustering of genes involved in transfer and replication, the insertion of a transposable element within either of these segments is likely to cause loss of an important or essential function. Plasmids carrying such insertions are therefore less likely to survive than those in which the transposition event occurred within a non-essential region. Once acquired, much of the transposable element itself would then be non-essential to the plasmid and so would become a favoured region for insertions of additional elements. A series of such transpositions would lead to the apparent clustering of antibiotic resistance determinants in the same region of the plasmid, and in some

cases the transposability of certain determinants might be abolished by subsequent insertions, or by transposition-mediated deletions.

Successive transpositions of this kind might account for the arrangement of interspersed transposable elements in the resistance region of IncFII plasmids, (e.g. see Brevet et al, 1977) and is also consistent with data presented in Chapter 7 for R46 and N3. The resistance regions of both these plasmids include a pair of 'ISN' insertion sequences, and the Ap^R and Spc^R determinants of R46 probably lie within a transposon. Moreover, a number of inverted repeat sequences have been detected on both plasmids, and even if these are not presently components of active transposable elements, they may have been so in the past.

The genetic organisation of the IncP plasmid RK2 (or RP1, or RP4) differs markedly from that of plasmids in other groups, in that the DNA essential for replication is contained in two distinct regions, and similarly the conjugal transfer functions are encoded by at least two different segments. The intervening blocks of DNA in each case include an antibiotic resistance determinant, and it is possible to interpret this structure as the result of transpositional insertions within the replication and transfer regions of an ancestral plasmid, which in this case did not seriously impair either function.

Plasmids of the incompatibility group N have attracted interest because of their relatively wide host-range, their highly efficient conjugation mechanism on solid surfaces, the IncN-specific restriction and modification system EcoRII, and the UV-protection and mutagenesis phenotypes determined by several members of the group. As well as providing material for comparative studies of plasmid organisation, the data presented in this thesis will form the basis of more detailed studies of the representative IncN plasmids R46 and N3. The nature

of the transposable elements present on these plasmids, and the genetic organisation of their transfer regions are two of several aspects which merit further investigation.

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A Physical and Genetic Map of the IncN Plasmid R46

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Received November 21, 1980

A combined physical and genetic map of the conjugative IncN plasmid R46 was obtained by restriction endonuclease cleavage analysis, followed by the construction and analysis of deletion and recombinant derivatives. The genetic determinants for the antibiotic resistance and uv-protection phenotypes were located, as well as the regions necessary for plasmid replication and for conjugal transfer. The end points of the deletion giving rise to the R46 derivative pKM101 were localized.

The conjugative antibiotic-resistance plasmid R46 was one of the first plasmids identified as a member of incompatibility group N, the third major incompatibility group to be recognized in *E. coli* (Datta and Hedges, 1971). IncN plasmids have a host range that includes many genera of the *Enterobacteriaceae* (Jacob *et al.*, 1977; Tardif and Grant, 1980), and it has been reported that R46 is also transmissible to *Pseudomonas* (Jacoby, 1975, 1977).

R46 confers resistance to the antibiotics tetracycline, spectinomycin (and streptomycin at low concentration), sulfonamides, and ampicillin; the β -lactamase gene is of the oxacillin-hydrolyzing type OXA-2 (Matthew and Hedges, 1976). R46 also determines resistance to arsenate ions (Summers *et al.*, 1978). The plasmid is self-transmissible at high efficiency on solid media, though poorly in liquid matings (Dennison and Baumberg, 1975). It confers sensitivity to the IncN-specific filamentous bacteriophage Ike (Khatoon *et al.*, 1972), as well as to the phage PR4, which is specific for cells containing plasmids of Inc groups N, P, and W (Bradley and Rutherford, 1975). Bradley (1979) has shown that cells carrying an IncN plasmid produce short nonflexible pili, to which the phages Ike and PR4 will adsorb. IncN plasmids also exhibit fertility inhibition relationships with plasmids of Inc groups P, W, and X (Olsen and Shipley, 1975).

In addition, R46 protects its host cell from the lethal effects of uv irradiation (Drabble and Stocker, 1968). This is one of several plasmid-borne phenotypes related to the repair of uv damage expressed in *E. coli* and *S. typhimurium* by both R46 and a deletion derivative of R46 called pKM101 (Mortelmans and Stocker, 1979). As well as protection against uv and other DNA-damaging agents, both these plasmids cause enhanced uv-induced and chemical mutagenesis, and an increased spontaneous mutation rate (Mortelmans and Stocker, 1976, 1979; Walker, 1977). It is likely that all these effects are mediated by a single gene product (Walker, 1978; Shanabruch and Walker, 1980), which probably increases the host cell's capacity for error-prone repair (Mortelmans and Stocker, 1976; Walker and Dobson, 1979). The enhanced chemical mutagenesis mediated by pKM101 has led to the incorporation of this plasmid in the Ames test for mutagens, where it renders the *Salmonella* test strains more susceptible to mutation by a variety of compounds (McCann *et al.*, 1975).

In this paper we present a physical and genetic map of R46 based upon restriction endonuclease cleavage analysis. The locations of the genetic determinants of the plasmid phenotypes have been deduced from the phenotypes of (i) R46 derivatives with *in vitro* deletions of specific restriction fragments, (ii) plasmids with deletions ex-

tending from a single restriction site, and (iii) recombinant clones of specific R46 restriction fragments in pBR325. In addition the limits of the deletion carried by pKM101 have been localized.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Phages

E. coli K-12 strains were ED395 (*lac* Δ U-124; Dempsey and Willetts, 1976), ED3818 (*his lys trp str nal*; Willetts *et al.*, 1976), ED8654 (*met*; Borck *et al.*, 1976), and AB1157 (*arg his leu pro thr str*; Howard-Flanders and Theriot, 1966).

The plasmids were R46 (Ap^RAs^RSpc^RSul^RTc^RUvp⁺Tra⁺; Datta & Hedges, 1971), pKM101 (Ap^RUvp⁺Tra⁺; Mortelmans and Stocker, 1979), pSC101 (Cohen and Chang, 1977), and pBR325 (Bolivar, 1978).

The bacteriophages used were Ike (Khattoon *et al.*, 1972) and PR4 (Bradley and Rutherford, 1975), and were obtained from S. Baumberg and J. Watson, respectively.

Media

The culture media were described by Willetts and Finnegan (1970). Antibiotics were added to Oxoid nutrient agar to give the following concentrations: ampicillin (40 μ g/ml), chloramphenicol succinate (50 μ g/ml), spectinomycin (20 μ g/ml), and tetracycline (20 μ g/ml). In minimal media the concentrations were ampicillin (50 μ g/ml), chloramphenicol succinate (50 μ g/ml), spectinomycin (100 μ g/ml), streptomycin (500 μ g/ml), sulfadimidine (100 μ g/ml), tetracycline (10 μ g/ml), and nalidixic acid (40 μ g/ml).

Genetic Techniques

Quantitative membrane filter matings were carried out by growing cultures of donor and recipient strains to 2×10^8 cells/ml; mixing 0.2 ml of the donor cell culture with 1.8 ml of the recipient, and filtering through a 10-mm-

diameter membrane filter (Millipore Corporation, pore size 0.22 μ m). The filter was then transferred to a prewarmed Oxoid nutrient agar plate and incubated for 30 min at 37°C. The cells were resuspended in 2 ml of L broth and appropriate dilutions were plated on selective media. Plate matings were carried out by spreading 0.1 ml of an appropriate dilution of a fresh overnight culture of the donor strain with 0.1 ml of a similar culture of the recipient strain directly on the selective medium.

ED8654 was transformed by a technique essentially similar to that of Lederberg and Cohen (1974).

Resistance of plasmid-containing cells to arsenate ions was determined by spotting approximately 10^3 cells onto nutrient agar containing 4 mg/ml sodium arsenate, incubating overnight at 37°C, and comparing their growth with that of control strains with and without R46.

uv Irradiation

Bacterial cultures were grown to 2×10^8 cells/ml, chilled, and diluted 100-fold in buffer; 5 ml of cell suspension were transferred to a glass petri dish and exposed to doses of 0, 25, 50, 75, and 100 J m⁻² of uv light with gentle agitation during irradiation; uv fluences were measured with a Laterjet uv meter. Appropriate dilutions of the irradiated cells were plated on nutrient agar and incubated overnight at 37°C.

Plasmid DNA Preparations

Plasmid DNA was prepared by a procedure based upon the cleared lysate technique of Clewell and Helinski (1970) and the polyethylene glycol precipitation technique of Humphreys *et al.* (1975). It was then purified by CsCl-ethidium bromide density gradient centrifugation.

Small-scale cleared lysates, used for rapid screening of plasmid sizes by gel electrophoresis, were prepared as described by Willetts *et al.* (1981).

Restriction Enzyme Techniques and Gel Electrophoresis

Restriction enzymes were purchased from Boehringer Mannheim GmbH, New England Biolabs Inc., and Bethesda Research Laboratories Inc., and were used in buffers similar to those recommended by the suppliers. *Hind*III and T4 ligase were gifts of K. Murray and *Pst*I was prepared in this laboratory. The sizes of DNA restriction fragments were determined after electrophoresis through 0.7% agarose slab gels, as described by Willetts and McIntire (1978). *Hind*III fragments of lambda (Daniels *et al.*, 1980) were used as molecular-weight standards.

End labeling of *Eco*RI and *Hind*III digestion products with [³²P]dTTP was by incubation with Klenow polymerase (Boehringer Mannheim GmbH). Radiolabeled fragments were separated by electrophoresis through 8% polyacrylamide gels (Maniatis *et al.*, 1975); the gels were then dried down and autoradiographed.

Measurement of Plasmid Size by Electron Microscopy

Plasmid molecules were spread and mounted on parlodion grids using the formamide technique of Davis *et al.* (1971). Double-stranded circular molecules of ϕ X174 (5.38 kb)¹ were added to provide length standards. Molecules were photographed using a Siemens Elmiskop 101 electron microscope. Negatives were projected and the outline of molecules traced and measured with a Keuffel and Esser map measurer.

RESULTS

(i) Length of the R46 Molecule

Open circular molecules of R46 DNA were photographed in the electron microscope; and their total lengths measured. Similar measurements were made of double-stranded circular ϕ X174 RF molecules on

the same grid, and comparison of the lengths allowed the size of R46 to be calculated as 51.7 ± 0.2 kb (mean of 11 molecules). By summing the sizes of the *Bgl*II + *Kpn*I double-digest fragments of R46 (see below) a further estimate of the total size of the plasmid was obtained (52.2 kb). This is closely similar to the size determined using the electron microscope, and in this paper the value of 51.7 kb is used.

(ii) Restriction Endonuclease Cleavage Map

The sizes of the cleavage fragments of R46 produced by the enzymes *Eco*RI, *Hind*III, *Bam*HI, *Bgl*II, *Sal*I, *Kpn*I, *Pst*I, *Sma*I, and *Xho*I were determined by agarose gel electrophoresis. Fragments of less than 0.35 kb would usually not have been detected. However, for the enzymes *Eco*RI and *Hind*III (and for double digests with these), the fragments were also analyzed by polyacrylamide gel electrophoresis of ³²P-end-labeled R46 DNA followed by autoradiography. This technique allows detection of fragments down to about 30 base pairs.

The locations of the cleavage fragments for each of the restriction endonucleases on the circular R46 map were then found by carrying out the appropriate series of double digestions, and comparing the fragments with those obtained using each enzyme separately. Most of the fragments were mapped by this method. For some fragments, however, it was necessary to supplement this data later, with further information derived from cleavage of R46 deletion plasmids and of recombinant clones containing specific R46 fragments. For example, the order of the fragments *Bgl*II-I, E, C, and F was determined by *Bgl*II digestion of a series of deletion plasmids of decreasing size (pED940-946; Table 1, Fig. 2a).

The resultant physical map of R46 is shown in Fig. 1. One peculiarity of note is that a large region of the plasmid contained many fewer cleavage sites than would be predicted on a random basis, and the majority of sites were clustered in one or perhaps two relatively small regions of the genome.

¹ Abbreviations used: kb, kilobase; Uvp, ultraviolet protection phenotype.

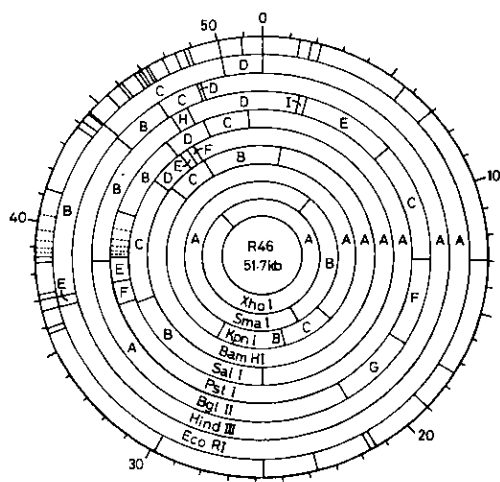


FIG. 1. Restriction endonuclease cleavage map of R46 for the enzymes *EcoRI*, *HindIII*, *BglII*, *PstI*, *SalI*, *BamHI*, *KpnI*, *SmaI*, and *XhoI*. The outer circle shows the sites for all nine enzymes. Fragments produced by each enzyme are labeled alphabetically in order of decreasing size. Dotted lines indicate the approximate location of six small *PstI* fragments, the order of which was not determined. Coordinates are marked in kilobases.

Of the nine restriction endonucleases we used, only *BglII* has an evenly distributed series of cleavage sites.

Comparison of the restriction enzyme cleavage fragments of R46 with those of its deletion derivative pKM101 showed that only fragments contiguous on our map were missing in the latter. We conclude that pKM101 was formed from R46 by a single deletion event. The endpoints of this deletion were deduced to lie between *SalI* (coordinate 35.8 kb) and *EcoRI* (36.6 kb) sites at one end, and between two *EcoRI* sites (50.05 and 51.7 kb) at the other end (see Fig. 4). Moreover, each endpoint lies close to one of the *PstI* sites at coordinates 35.95 and 50.9 kb, respectively, in R46 (Fig. 4). At least one of these *PstI* sites was retained in pKM101 and if both are present they lie less than 0.3 kb apart (data not shown).

(iii) Construction of *in Vitro* Deletions and Rearrangements of R46

Restriction enzyme digests of R46 DNA produced by one of the five enzymes *EcoRI*,

HindIII, *BamHI*, *BglII*, or *SalI* were ligated and used to transform ED8654 with selection for each in turn of the Ap^R , Spc^R , Sul^R , and Tc^R phenotypes of R46. The rationale for these experiments was that the one or more fragments carrying the antibiotic resistance gene would in some cases be ligated to the fragment(s) of R46 containing the region essential for plasmid replication, and the combination therefore recovered as a new plasmid.

When *HindIII* and *SalI* were used in this procedure with selection for Spc^R transformants, the plasmids recovered (pED904 and pED923 are representatives; see Table 1) were composed of single cleavage fragments. The Spc^R gene and the replication region of R46 are therefore on the same *HindIII* and *SalI* fragments. The large *HindIII*-A fragment also carries the Ap^R and As^R genes, while the *SalI*-A fragment carries the Ap^R and Sul^R genes in addition to that for Spc^R (Table 1).

In all other cases, the recombinant plasmids contained two or more fragments. One fragment was common to all of the recombinant plasmids generated by a particular enzyme, and it was inferred that this fragment carried the replication region. The location of this region will be discussed in more detail below. The additional fragment(s) varied according to the antibiotic resistance phenotype selected, and this provided preliminary information about the locations of the genes determining these phenotypes. The restriction fragment composition and phenotypes of the recombinant plasmids are listed in Table 1. The fragments were not always in the same order or orientation as in R46, and some of the recombinant plasmids (e.g., pED917) contained gratuitous fragments not initially selected.

(iv) Isolation of Deletions from the *HindIII* Site of pED904

When linear plasmid DNA molecules generated by a restriction endonuclease are used to transform *E. coli* cells, transformants

TABLE 1
PLASMIDS DERIVED FROM R46

Plasmid	Composition/size	Phenotype ^a							
		Ap	Spc	Sul	Tc	As	Uvp	Ike, PR4 ^b	Tra
(a) <i>In vitro</i> deletions and rearrangements									
pED904	<i>Hind</i> III-A	R	R	S	S	R	+	S	+
pED905	<i>Hind</i> III-A,B	R	R	S	R			S	
pED906	<i>Hind</i> III-A,C	R	R	R	S			S	+
pED907	<i>Bam</i> HI-A,B	R	R	R	S			S	+
pED912	<i>Eco</i> RI-A,B	S	S	S	R	R	+	S	+
pED918	<i>Eco</i> RI-A,D	R	R	S	S	S		S	+
pED923	<i>Sal</i> I-A	R	R	R	S	S		R	-
pED910	<i>Bgl</i> II-D,E,G	R	R	S	S		-	R	-
pED911	<i>Bgl</i> II-B,E,G	S	S	S	R		-	R	-
pED915	<i>Bgl</i> II-D,E	R	R	S	S	S		R	-
pED916	<i>Bgl</i> II-B,E	S	S	S	R	S	-	R	-
pED917	<i>Bgl</i> II-B,C,D,E,G,I	R	R	S	R		-	R	-
pED919	<i>Bgl</i> II-A,B,C,D,E,F,G	R	R	S	R			S	± ^c
pED920	<i>Bgl</i> II-B,D,E,G,I	R	R	S	R			R	-
(b) Deletions of pED904									
pED940	21.3 kb	R	R			S	+	R	-
pED941	16.8 kb	R	R					R	-
pED942	15.4 kb	R	R						-
pED943	14.3 kb	R	R				+		-
pED944	11.2 kb	R	R						-
pED945	10.8 kb	R	R				+		-
pED946	9.7 kb	R	R				-		-
pED949	6.9 kb	R	S				-		-
(c) Recombinant clones ^d									
pED926	pBR325 + <i>Bgl</i> II-H	R	S	S	S			R	
pED927	pBR325 + <i>Bgl</i> II-A	R	S	S	S	S		S	-
pED928	pBR325 + <i>Bgl</i> II-F	R	S	S	S			R	
pED929	pBR325 + <i>Bgl</i> II-D	R	R	S	S			R	
pED930	pBR325 + <i>Bgl</i> II-G	R	S		S				
pED935	pBR325 + <i>Pst</i> I-A of pED953	S	S		R			S	+
(d) Transposon derivative									
pED953 ^d	R46::Tn5	R	R		R			S	+

^a Abbreviations: Ap, ampicillin; Spc, spectinomycin; Sul, sulfonamide; Tc, tetracycline; As, arsenate; Uvp, ultraviolet protection; Tra, transfer. A blank space in the table indicates that the relevant test was not performed.

^b Sensitivity to Ike and PR4 was determined by spotting 0.01 ml of phage suspension on a lawn of bacteria poured in LC top agar.

^c pED919 was self-transmissible at approximately 1% of the frequency of R46: rearrangement of the *Bgl*II fragments was responsible for this decrease.

^d In addition to the phenotypes listed, all the pBR325 recombinant derivatives also conferred resistance to chloramphenicol (Cm), and pED953 conferred resistance to kanamycin (Km).

are recovered at a very low frequency, and frequently contain plasmids that have suffered deletions of random length extending from one or both ends of the linear molecule (Thompson and Achtman, 1979).

We used this technique to generate a series of random deletions of pED904 from its single *Hind*III site. pED904 is a recombinant plasmid containing only the *Hind*III-A fragment of R46, and determines resistance to ampicillin and spectinomycin (see Table 1). Preliminary experiments using R46 showed that linear molecules with *Hind*III cohesive ends could be precisely religated *in vivo* by *E. coli* regenerating a (new) *Hind*III site and a plasmid similar to pED904. To avoid this, pED904 molecules linearized with *Hind*III were treated with alkaline phosphatase to remove the 5' P termini, before being used for transformation. Ap^R and Spc^R transformants were each found at a frequency of about 10 per microgram of plasmid DNA.

The sizes of the plasmids in the transformant clones were measured by agarose gel electrophoresis of small-scale cleared lysate preparations. A range of plasmids of different sizes was then chosen for further study, from the smallest (pED949 = 6.9 kb) to the largest that was clearly smaller than pED904 (pED940 = 21.3 kb). Restriction enzyme analysis confirmed that the plasmids were derivatives of pED904 deleted to varying extents from the *Hind*III site, and helped to locate the endpoints of the deletions. As was expected from the proximity of the Spc^R and Ap^R markers to one end of the *Hind*III-linearized molecules, the larger deletions were always of the other end of the molecules.

(v) Cloning the R46 *Bgl*II Fragments into pBR325

To facilitate correlation of the genetic and physical maps of R46, several of its nine *Bgl*II fragments were cloned into pBR325. *Bgl*II was the only restriction endonuclease tested that cleaved R46 at a fairly evenly

distributed set of sites, the largest *Bgl*II fragment being 17.1 kb long. It was possible to clone these fragments into the *Bam*HI site of pBR325 since both enzymes produce 5'-GATC single-strand extensions.

R46 DNA was cleaved with *Bgl*II and mixed with pBR325 DNA cleaved with *Bam*HI, and the mixture was ligated. ED8654 was transformed with the resultant molecules, and Cm^R transformants were selected. Clones carrying this pBR325 marker were then screened to identify those that were Tc^S: insertion of DNA fragments into the *Bam*HI site of pBR325 inactivates this gene. Small-scale cleared lysate plasmid preparations were made from 37 such Cm^RTc^S clones, and the approximate sizes of the plasmid molecules were measured by agarose gel electrophoresis to allow representative plasmids carrying different R46 *Bgl*II fragments to be chosen for further study. Subsequent restriction enzyme analysis of these showed that *Bgl*II fragments A, D, F, G, and H had been cloned, and these plasmids were numbered pED926 to pED930. Their phenotypes are listed in Table 1 and some are further discussed below.

(vi) Location of the Replication Region

The region of the R46 molecule which is essential for plasmid maintenance and replication was identified as that region of DNA which is common to all the deletion derivatives constructed. When the restriction endonuclease *Bgl*II was used to generate *in vitro* deletions and rearrangements of R46 (see section iii), plasmids of several different sizes were obtained, examples of which are pED910, 911, 915, 916, 917, 919, and 920. *Bgl*II restriction analysis of these plasmids revealed that all of them were composed of two or more *Bgl*II fragments, one of which was always *Bgl*II-E (Table 1). No other *Bgl*II fragment was common to all seven plasmids, and the *in vitro* deletions of R46 constructed using other restriction enzymes all contained the DNA corresponding to this fragment. This strongly implies that

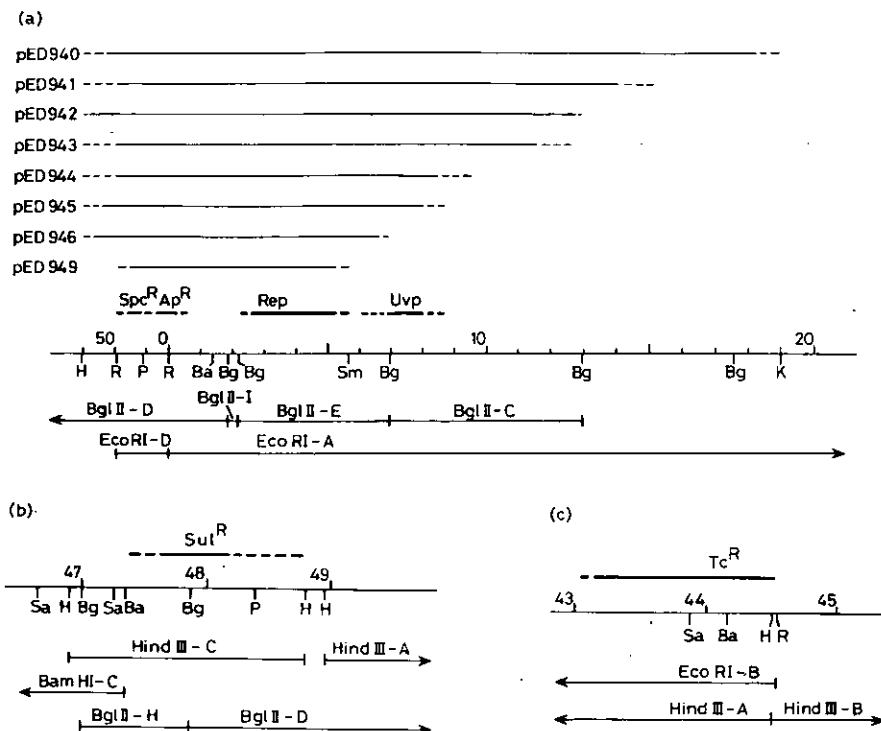


FIG. 2. Expanded maps of three regions of R46. (a) Map of R46 from coordinates 49 to 20 kb showing the lengths of DNA contained in deletion plasmids derived from pED904, the location of the replication region (*Rep*), and the positions of the genetic determinants for ampicillin resistance, spectinomycin resistance, and uv protection. (b) The sulfonamide resistance region. (c) The tetracycline resistance region. Dotted lines indicate uncertainty of the precise endpoints of deletion plasmids and phenotypic determinants (see text for details). Restriction cleavage fragments relevant to the text are shown; arrowheads indicate that a fragment extends off the figure. Coordinates are marked in kilobases. Key to restriction enzyme sites: *R*, *EcoRI*; *H*, *HindIII*; *Bg*, *BglII*; *P*, *PstI*; *Sa*, *SaI*; *Ba*, *BamHI*; *K*, *KpnI*; *Sm*, *SmaI*; *X*, *XhoI*.

the essential replication functions and origin of replication of R46 are located entirely within the 4.75 kb *BglII*-E fragment (Fig. 2a): The fragment carries no antibiotic-resistance markers so that in the production of *in vitro* deletions and rearrangements by *BglII* digestion at least one other fragment was always found ligated to *BglII*-E to provide a selectable phenotype.

The location of the essential replication region in this part of the R46 molecule was confirmed by restriction enzyme analysis of the deletions derived from pED904 (see section iv), designated pED940-946 and pED949 (Fig. 2a). Although these plasmids have all suffered extensive deletions anticlockwise

from the *HindIII* site in pED904, they all contain R46 DNA from approximately coordinates 50.5 to 5.6 kb (see Fig. 2a). This common region of DNA includes the *Ap^R* determinant and a part of the *BglII*-E fragment, thus serving to localize the segment essential for plasmid replication to within a region of approximately 3.4 kb of *BglII*-E, from coordinate 2.2 to approximately 5.6 kb (Fig. 2a). An origin of replication is presumably located within this segment. The smallest pED904 deletion plasmid recovered was pED949; this plasmid was stably maintained in nonselective media and expressed *IncN* incompatibility. pED949 constitutes a 6.9-kb *Ap^R* mini-R46 replicon.

(vii) *Location of the Antibiotic-Resistance Determinants*

(a) *Ampicillin resistance and spectinomycin resistance.* The *in vitro* deletion plasmid pED918 is composed of R46 fragments *EcoRI*-A and *EcoRI*-D, and conferred resistance to Ap and Spc (see Table 1). pED912, however, which is composed of fragments *EcoRI*-A and *EcoRI*-B, conferred resistance to Tc and As (Table 1). These data together imply that the *EcoRI*-D fragment carries both the Ap^R and Spc^R determinants, although part of one of these genes may lie on *EcoRI*-A.

Restriction enzyme analysis of the pED904 deletion derivatives pED946 and pED949 (Fig. 2a) showed that whereas the Ap^RSpc^R plasmid pED946 contained all of fragment *EcoRI*-D, pED949 which determined Ap^R only (Table 1) was deleted for part of this fragment. This establishes that the order of the Ap^R and Spc^R determinants in *EcoRI*-D is that shown in Fig. 2a. The Spc^R gene must map entirely within the limits of this fragment, while the Ap^R gene may extend into *EcoRI*-A.

(b) *Sulfonamide resistance.* Comparison of the *in vitro* deletion plasmids pED904 (*HindIII*-A fragment alone) and pED906 (*HindIII*-A + *HindIII*-C; see Table 1) shows that the additional presence of the *HindIII*-C fragment imparted Sul^R to the pED906 phenotype. Moreover, since the *HindIII*-A and *HindIII*-C fragments are not contiguous in the parent R46 molecule (Fig. 1), the Sul^R determinant is most likely located entirely within *HindIII*-C.

The Sul^R gene(s) can be more precisely delimited within this fragment. The plasmid pED907 (Table 1) is deleted for the fragment *Bam*HI-C, i.e., for the DNA immediately to the left of the *Bam*HI site in *HindIII*-C as shown in Fig. 2b, but retained the Sul^R phenotype. pED915 is a plasmid composed of the fragments *Bgl*II-D and *Bgl*II-E, and pED929 is a recombinant clone of *Bgl*II-D in pBR325 (Table 1). Both these plasmids there-

fore contain the DNA to the right of the right-hand *Bgl*II site in *HindIII*-C (Fig. 2b). Neither of these plasmids conferred the Sul^R phenotype (Table 1). These data position one end at least of the Sul^R determinant between these *Bam*HI and *Bgl*II sites. The other end must lie to the right of the *Bgl*II site since Sul^R is not expressed by pED926, a recombinant clone of the *Bgl*II-H fragment in pBR325 (Table 1, Fig. 2b). The region of R46 DNA that includes the entire Sul^R gene(s) is therefore bounded by *Bam*HI and *HindIII* sites which lie 1.4 kb apart (Fig. 2b). This region contains single cleavage sites for *Bgl*II and *Pst*I, and the *Bgl*II site must lie within the Sul^R determinant.

(c) *Tetracycline resistance.* The Tc^R plasmid pED912 is composed of R46 fragments *EcoRI*-A and *EcoRI*-B (Table 1) which are not contiguous in the parent R46 molecule (Fig. 1). pED918 is composed of *EcoRI*-A and *EcoRI*-D and was Tc^S (Table 1). These data imply that the Tc^R determinant of pED912 is located within *EcoRI*-B. Tc^R was not expressed from the *Hind*-A fragment in pED904, although this contains all of *EcoRI*-B except the small terminal *HindIII*-*EcoRI* segment, measured as approximately 30 base pairs on polyacrylamide gels (Table 1, Fig. 1). This fixes one end of the Tc^R determinant between the *HindIII* site and adjacent *EcoRI* site at the extreme right hand end of *EcoRI*-B (Fig. 2c). As expected, pED905, consisting of the fragments *HindIII*-A and *HindIII*-B ligated in their original orientation, did confer Tc^R (Table 1).

The restriction map of this region R46 is identical to that of the Tc^R gene(s) of the plasmid pSC101 (Tait and Boyer, 1978) as shown by the comigration of the *EcoRI*-*HindIII*, *HindIII*-*Bam*HI, and *HindIII*-*Sal*I double-digest fragments on polyacrylamide gels. Assuming that the Tc^R determinants are of similar size, we therefore infer that the left-hand end of the R46 Tc^R determinant is located at approximately coordinate 43.1 kb.

(d) *Arsenate resistance.* The R46 *in vitro* deletion derivatives pED912 and pED918 are composed of *EcoRI* fragments A + B,

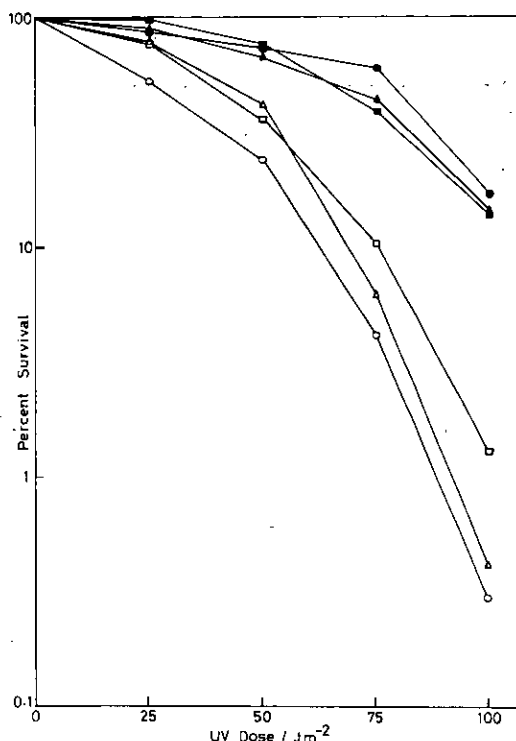


FIG. 3. Ultraviolet dosage-survival curves of AB1157 strains containing R46 derivatives ●, R46; ■, pED912; ▲, pED945; ○, no plasmid; △, ED946; □, pED917.

and A + D, respectively (Table 1). pED912 determined resistance to arsenate ions while pED918 did not. Since *EcoRI*-A and *EcoRI*-B are not contiguous in the R46 molecule (Fig. 1), these data indicate that the genetic determinant for As^R lies on *EcoRI*-B. This fragment contains a single site for the enzyme *Bgl*II (see Fig. 1) and it was possible to test each of *Bgl*II fragments bounded by this site (*Bgl*II-A and *Bgl*II-B) for their ability to confer As^R using the plasmids pED927 and pED916. pED927 is a recombinant clone of *Bgl*II-A in pBR325 (Table 1) and pED916 is an *in vitro* recombinant composed of the *Bgl*II-B and *Bgl*II-E fragments only (Table 1). Neither of these two plasmids conferred resistance to arsenate ions so it was concluded that the *Bgl*II site between *Bgl*II-A and *Bgl*II-B lies within the As^R determinant region of R46 (see Fig. 4).

(viii) Location of the *Uvp* Region

The location of the plasmid gene(s) re-

sponsible for the uv-protection phenotype (*Uvp*) of R46 was determined by testing recombinant and deletion derivatives of R46 for their abilities to protect AB1157 against the lethal effects of uv irradiation. On the basis of uv dosage survival curves (Fig. 3) the plasmids tested clearly fell into two classes, judged to be *Uvp*⁺ and *Uvp*⁻ by reference to AB1157(R46) and AB1157 controls.

The *in vitro* deletion plasmid pED904 (Table 1) was found to be *Uvp*⁺. A number of the deletion mutants derived from this plasmid (section (iv)) were therefore also tested. As shown in Fig. 2a, all of these pED904 derivatives (except pED949) have left-hand endpoints between the *Hind*III site at coordinate 48.95 kb and the *Eco*RI site at 50.05 kb, and contain DNA extending to the right for various distances. The smallest of these plasmids that still conferred uv-protection is pED945 (Fig. 2a, Fig. 3). The R46 *in vitro* deletion plasmid pED912 (Table 1) was *Uvp*⁺ (Fig. 3) and is deleted for the DNA extending leftward from 0 kb to beyond the left-hand endpoint of pED945 as drawn in Fig. 2a. Consequently the *Uvp* determinant must be between 0 kb and the right-hand endpoint of pED945 at approximately 8.7 kb.

The largest of the *Uvp*⁻ pED904 deletion derivatives was pED946 (Fig. 2a, Fig. 3) which is 1.05 kb smaller than the *Uvp*⁺ plasmid pED945. This further localizes an essential part of the *Uvp* determinant between the right-hand endpoints of pED945 and pED946 as shown in Fig. 2a, a region which includes the *Bgl*II site at the junction of fragments *Bgl*II-E and *Bgl*II-C.

The recombinant plasmid pED917 was constructed by ligation of a *Bgl*II digest of R46, and is composed of *Bgl*II fragments B, C, D, E, G, and I (Table 1). It therefore contains all the DNA present in pED945, though with the fragments religated in an order different to that in the R46 molecule. pED917 was *Uvp*⁻ (Fig. 3); this implies that the *Uvp* determinant is present on more than one *Bgl*II fragment, and that the order of *Bgl*II-E and *Bgl*II-C had been rearranged

sary for transfer cloned on a DNA fragment smaller than *EcoRI*-A, the Km^R transposon Tn5 was used to provide a suitably placed *PstI* restriction site. A series of R46::Tn5 plasmids was constructed, and the positions of the transposons were mapped by restriction analysis. The properties of these plasmids will be described in detail elsewhere. pED953 is one such plasmid, with Tn5 inserted within *EcoRI*-A at coordinate 13.45 kb (Fig. 4), and was self-transmissible at the same frequency as R46. For the cloning experiment we made the assumption that the genes required for transfer are clustered between the Tn5 insertion in pED953 and the *PstI* site at coordinate 35.95 kb, close to one end of *EcoRI*-A. The entire transfer region would then be located on a single *PstI* fragment of pED953.

PstI restriction digests of pED953 and pBR325 DNA were ligated and used to transform ED8654. Tc^R transformants were selected in broth containing tetracycline and the cultures mated with the Nal^R strain ED3818 using the efficient membrane filter technique. $Tc^R[Nal^R]$ transconjugants were selected and then tested for resistance to chloramphenicol and sensitivity to ampicillin. Such transconjugants should be obtained if the entire transfer region from pED953 had been linked to the Tc^R and Cm^R markers of pBR325 by its insertion at the *PstI* site within the Ap^R gene of pBR325, to form self-transmissible recombinant plasmids. This was indeed the case and further analysis of three such $Tc^R Cm^R Ap^S[Nal^R]$ transconjugants showed them to contain plasmids consisting of the 23-kb *PstI* fragment from pED953 cloned into pBR325. One of these recombinant plasmids was designated pED935. This plasmid conferred sensitivity to the phages I ϕ 8 and PR4, and was self-transmissible at about 100% of the frequency of R46. The 23 kb of cloned DNA contains approximately 0.55 kb of Tn5 DNA and the R46 DNA from between coordinates 13.45 and 35.95 kb. These results therefore confirm that all the genes necessary for plasmid transfer are located within this region of

R46. pBR325 was not mobilized by R46 so pED935 most probably also contains the R46 origin of transfer. The positions of Tn5 insertions in five other R46::Tn5 plasmids, which were transfer deficient (unpublished results), indicate that there are transfer genes located at about 3 kb from either end of the cloned R46 DNA segment in pED935. We do not yet know, however, if all the transfer genes form a single contiguous cluster or whether they are grouped in two or more distinct regions within this segment.

DISCUSSION

The construction and analysis of deletion and recombinant derivatives of the IncN plasmid R46, taken in conjunction with a restriction endonuclease cleavage map of the molecule, has allowed the production of a combined physical and genetic map (Fig. 4). The genetic determinants for the Ap^R , As^R , Spc^R , Sul^R , Tc^R , and Uvp phenotypes were located on the restriction cleavage map, as well as the regions responsible for plasmid replication and for conjugal transfer. These locations are consistent with the $Ap^R Uvp^+ Tra^+$ phenotype of the R46 deletion derivative pKM101, given the map position of the deleted region (Fig. 4). P. Langer and G. Walker (in preparation) have recently constructed a restriction cleavage map of pKM101 and their data for this part of the R46 molecule are in accord with ours. Previously Shanabruch and Walker (1980) had located a region necessary for the Uvp and related phenotypes of pKM101 within a 2.2-kb segment which corresponds to that between coordinates 5.65 and 7.85 kb on our map of R46; this is consistent with our location of the Uvp determinant (Fig. 2a).

We found that a single region of approximately 3.4 kb of the R46 molecule contained the only R46 DNA required to support plasmid maintenance and replication, and an Ap^R mini-R46 replicon (pED949) of 6.9 kb, which includes this region, was constructed. A single essential replication region has been observed for a number of other con-

jugative plasmids, e.g., F (Lovett and Helinski, 1976) and R6-5 (Timmis *et al.*, 1975), although replication of the broad host-range IncP plasmid RK2 (Thomas *et al.*, 1980) requires three well-separated segments of the molecule.

The restriction enzyme cleavage patterns of the regions of R46 identified as determining the Sul^R and Tc^R phenotypes were found to show striking similarities with the equivalent resistance regions of other plasmids. The Sul^R determinant of R46 was deduced to lie within a 1.4-kb region bounded by *Bam*HI and *Hind*III sites and containing sites for *Bgl*II and *Pst*I. A similar restriction pattern of *Bam*HI–*Bgl*II–*Pst*I–*Hind*III sites is observed in the Sul^R regions of the IncW plasmids R388 and Sa (Ward and Grinsted, 1978, and personal communication) and of another IncN plasmid, N3 (our unpublished results); it seems likely that these genes may be homologous. The restriction cleavage pattern of the Tc^R region of R46 was found to be indistinguishable from that of the equivalent region of pSC101 (see section (vii)(c)). This strongly suggests that R46 carries a Class-C tetracycline resistance determinant as defined by Mendez *et al.* (1980).

The overall distribution of hexanucleotide-specific restriction enzyme cleavage sites around the R46 molecule was observed to be strongly asymmetric in that a large region (approximately 35 kb) of the molecule has many fewer cleavage sites than would be expected on a random basis for nine different restriction enzymes. This region was shown to contain the DNA required for plasmid maintenance and replication, for conjugal transfer, and for the Uvp phenotype. The majority of restriction sites present in the R46 molecule were located close to or within regions responsible for the five antibiotic resistance phenotypes. A similar scarcity of cleavage sites in regions not concerned with antibiotic resistance genes has been observed in plasmids of incompatibility groups W, P, and Q, and may be a common feature among plasmids with broad host ranges

(Ward and Grinsted, 1978; Thomas *et al.*, 1980; Barth, 1979). It has been suggested (Barth, 1979; Thomas *et al.*, 1980) that this phenomenon may be the result of selection for the loss of cleavage sites in broad host range plasmids, and that the more numerous sites around the resistance markers may reflect the relatively recent acquisition of these genes in the evolution of the plasmids. Transposition would provide a plausible mechanism for the acquisition of resistance genes, and we are currently searching for transposable elements on IncN plasmids.

The two conjugative plasmid transfer systems that have been most fully characterized to date are those of the IncF plasmids (e.g., F) and the IncP plasmids (e.g., RP4). The organizations of these transfer systems differ in that the F-transfer genes lie in a single contiguous cluster of approximately 33 kb of DNA (reviewed by Willetts and Skurray, 1980), whereas in RP4 the transfer genes are clustered in two or three distinct regions, two of these being separated by an antibiotic resistance determinant (Barth *et al.*, 1978; Barth, 1979). By constructing a self-transmissible recombinant plasmid (pED935) containing all the R46 DNA necessary for transfer, we have localized the transfer genes of R46 to within a 22.5-kb region of the molecule which contains none of the other phenotypic determinants that we have mapped. The organization of the transfer genes within this region remains to be determined, and the recombinant plasmids we have constructed containing cloned fragments of this region should facilitate further genetic analysis of R46 conjugal transfer.

ACKNOWLEDGMENTS

We are grateful to Pam Beattie for her help with the electron microscopy, and to Graham Walker for communicating data prior to publication. This research was supported by MRC Programme Grant G978/88 to N.S.W. and by an MRC Research Studentship to A.M.C.B.

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