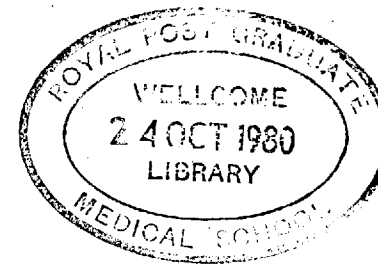
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THE ROLE OF BACTERIAL PLASMIDS IN GENETIC TRANSFER.

By

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ABSTRACT: Studies are described on the actual and potential ability of plasmids to transfer genes both within and between bacterial species. Comparisons between phenotypically-related antibiotic-resistance plasmids from a wide variety of bacterial hosts isolated in diverse geographical locations showed that a particular plasmid type, once evolved, could spread rapidly and widely in response to selective pressure. Plasmid transfer by conjugation and mobilisation was investigated using a series of insertion mutations in a conjugative Inc P plasmid by means of a transductional complementation system. At least five functions involved in P group conjugation were identified. The ability of plasmids to accept and transfer transposons was considered. Transposition by transposon 7 was characterised and a host function involved in transposition identified. Plasmid-mediated transfer of chromosomal genes was studied using R' plasmids generated *in vivo* and *in vitro*. The relative importance of extensive plasmid-chromosome homology, the *recA* gene and the insertion sequence IS1 were assessed and a general model proposed for chromosome mobilisation. Finally, interactions between plasmids and bacteriophages were considered. Transduction of plasmid DNA by bacteriophage P1 was shown to be a more complex process than earlier studies had indicated. The bacteriophage was shown to be capable of transducing a plasmid of 90Md. and a site-specific nuclease was shown to be involved in packaging plasmid DNA. A model is proposed for plasmid transduction.

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INTRODUCTION: THE DIVERSITY OF BACTERIAL PLASMIDS

The essential genes of many diverse bacterial species have been shown in each case to constitute a single linkage group or chromosome (see Novick, 1969). The fertility factor F in *Escherichia coli* was the first example of a stable, autonomous, extrachromosomal linkage group (plasmid) to be demonstrated (Hayes, 1953). It codes for no indispensible functions, all its known properties being connected with its maintenance, replication and transfer.

Plasmids coding for additional functions were first identified in Japan in the late 1950s, when multiple antibiotic resistance in Shigella was shown to be infectious and the genes involved to be separate from the chromosome (see Watanabe, 1963). Similar findings were soon reported in other parts of the world (see Datta, 1965) and plasmid borne antibiotic resistance is now known to be extremely common and to occur in many different bacterial genera (Table I.1).

Subsequently, many other plasmid borne characters have been observed, and some of these are listed in Table I.2. It is the central theme of this thesis that bacterial plasmids are of great importance in the spread of genetic information in the course of which any character may become plasmid borne in response to selective pressure. Plasmids can acquire new functions by interactions with other plasmids, bacterial chromosomes, transposons and bacteriophages, and then transfer the new property to other bacteria by conjugation or transduction. All of these aspects are considered in the following chapters.

Plasmids are thus dispensible, transmissible, extrachromosomal replicons which enlarge the gene pool of a bacterial population and provide sites where genetic "experiments" can be carried on without harming the host. This is advantageous because bacteria are haploid and chromosomal mutations are likely to affect viability. This view

Table I.1. The range and spread of plasmids coding for antibiotic resistance

Plasmids have been isolated coding for resistance to:

- Penicillins and cephalosporins
- Chloramphenicol
- Tetracyclines
- Erythromycin and other macrolides
- Streptomycin
- Neomycin/Kanamycin and related aminoglycosides
- Gentamicin, Tobramycin
- Amikacin
- Fusidic acid
- Sulphonamides
- Trimethoprim

Antibiotic resistance plasmids have been identified in:

- Enterobacteriaceae (many different genera)
- Psudomonas
- Vibrio
- Yersinia
- Pasteurella
- Haemophilus
- Neisseria
- Bacteroides
- Staphylococci
- Streptococci
- Clostridia
- Streptomyces

Table I.2. Plasmid-borne characteristics

Resistance genes:

Antibiotic resistance (see Table I.1)
Heavy metal resistance eg. arsenic, bismuth, lead, mercury, cadmium
Resistance to colicins
Resistance to female-specific phages.
Resistance to ultraviolet light by eg. R46, R2.

Utilisation of unusual carbon sources:

eg. Enzymes degrading camphor
Octane
Salicylate
Utilisation of sucrose and lactose.

Steps in major metabolic pathways:

eg. Degredation of "stable" RNA (by F)
DNA polymerase (R-Utrecht).

Enzymes for restriction and modification:

eg. *EcoRI* (RY13)
P1 endonuclease (P1)
DNA methylase (N3).

Functions associated with conjugation:

eg. Transmissibility
Sensitivity to male specific phages
Surface exclusion.

Production of antibacterial agents:

eg. Colicins (various Col factors)
Bacteriocins (*Streptococcus faecalis*)
Antibiotics (SCP1 in *S. coelicolor*).

Virulence factors:

eg. K88 antigen
Enterotoxin production.

Miscellaneous functions:

Inhibition of certain phages
Sensitivity to silicate
Sensitivity to sodium dodecyl sulphate
Sensitivity to acridines
Tumorgenicity (Ti plasmid in *Agrobacterium tumefaciens*).

The data in this Table are from Novick, 1969; Cohen, 1976 and Reanney, 1976.

of plasmids is supported by the observation of cryptic plasmids which confer no known characteristics (Smith and Linggood, 1970; Gyles *et al.*, 1974).

In view of the remarkable ability of plasmids to bring about exchange of genetic information, their classification should be based on a more fundamental property than that of their conferred phenotypes. The phenomenon of plasmid incompatibility provides such a basis (Datta, 1975). Plasmid replication, whether positively or negatively regulated, involves specific proteins and/or cellular sites. When two plasmids of similar replication specificity are present together in the same cell, there is mutual interference with replication - whether through the simultaneous production of the same repressor (Pritchard *et al.*, 1969) or competition for the same cellular sites (Jacob *et al.*, 1963). This results in the eventual loss of one or other of the pair of incompatible plasmids. Compatible plasmids, on the other hand, can coexist stably in the same cell line for many generations. Recent work has suggested that the basis of incompatibility is more complex, involving negative regulation in some cases (Cabello *et al.*, 1976) and positive controls in others (Kolter *et al.*, 1978). Partition mechanisms which ensure that both daughters receive a plasmid copy at cell division are believed also to play a part (Novick and Schwesinger, 1976; Timmis *et al.*, 1978). However, incompatibility between a pair of plasmids may be taken as an indication of their fundamental relationship and has been used to place plasmids in groups, the members of which are incompatible with each other and compatible with members of all other groups. So far, over twenty groups have been identified among plasmids from *E. coli* and other enteric bacteria (see Jacob *et al.*, 1977a).

Therefore, although the number of different phenotypes conferred by plasmids may run into thousands, the number of basic plasmid types is

relatively small. The significance of this in relation to the possible origin of plasmids is discussed in Chapter 5.

The work presented here is intended to illustrate ways in which the versatility of bacterial plasmids has made a singular contribution to bacterial evolution.

CHAPTER 1

MATERIALS AND METHODS

Bacterial strains. Strains are listed in Table 1.1.

Bacterial plasmids. Plasmids are shown in Table 1.2.

Bacteriophages. Phages are listed in Table 1.3.

Nutrient media. Oxoid No. 2 broth (CM67)
Oxoid New Zealand agar (+ No. 2 broth)
Oxoid Isosensitest agar (CM471)
Oxoid MacConkey agar (CM76).

Minimal media. Mimimal salts agar (Clowes and Hayes, 1968) using

Oxoid No. 3 agar at 1.2%.
M9 (Roberts *et al.*, 1955).

Supplements to minimal media

Glucose, lactose and other carbon sources to a concentration of 0.2% weight/volume.
Amino acids to a concentration of 20 µg/ml.
Vitamins to a concentration of 1 µg/ml.

Buffers

TES buffer pH8

50 mM Tris
50 mM NaCl adjust pH with concentrated (10M) HCl
5 mM EDTA

TNE buffer pH7.5

50 mM Tris
50 mM NaCl adjust pH with concentrated (10M) HCl
1 mM EDTA

Borate buffer pH8.3

89 mM Tris
89 mM Boric acid
1.25 mM EDTA

Acetate buffer pH7.7 (Aaij and Borst, 1972)

40 mM Tris
20 mM Sodium acetate
2 mM EDTA

Buffers (cont)

Restriction enzyme buffers

1) For *Eco*RI and *Bgl*II digestions (pH 7.5):

1 M Tris
0.5 M NaCl
0.1 M $MgCl_2$

2) For *Hind*III digestions:

0.1 M $MgCl_2$.

Sterilisation. All minimal media, buffers and glucose solutions were sterilised at 121°C for 15 minutes. Amino acids and all other sugars were autoclaved at 110°C for 15 minutes. Antibiotics were made up aseptically using sterile water and stored at 4°C.

Antibiotics. The following antibiotics were used to select for resistant bacteria:

Antibiotic (abbreviation)	Selective concentration (µg/ml) in solid media
Ampicillin (Ap)	100
Chloramphenicol (Cm)	25
Gentamicin (Gm)	4
Kanamycin (Km)	25
Nalidixic acid (Nal)	25
Rifampicin (Rif)	50
Streptomycin (Sm)	15
Sulphonamide (Su)	100
Tetracycline (Tc)	10
Trimethoprim (Tp)	10

When selecting or testing for trimethoprim or sulphonamide resistance in the *thy* strain W3110T$^-$, both antibiotics were used at a concentration of 1000 µg/ml. in minimal salts agar.

All drugs were obtained from commercial sources except the following which were gifts: chloramphenicol pure powder from Parke Davis and Co.; trimethoprim lactate from the Wellcome Research Laboratories; nalidixic acid from Winthrop Laboratories; rifampicin from Ciba Laboratories.

Testing for antibiotic resistance. Oxoid "Multodiscs" (code 4997E) were used or antibiotic ditch plates prepared using the concentrations shown above. Tests for trimethoprim or sulphonamide resistance were done on

Oxoid Isosensitest agar (CM471) containing 4% lysed horse blood.

R plasmid transfer by conjugation

(a) Plate matings. A broth culture (generally of the antibiotic-sensitive recipient strain) was grown to 10^9 cells/ml. 0.1 ml was then spread over one half of a minimal plate containing the nutritional requirements of the recipient plus a selective concentration of an appropriate antibiotic. Colonies from a plate of the differentially marked donor (R^+) strain were streaked across the plate into the recipient using a loop. Plates were incubated at 37°C overnight. If the recipient was Nal or Rif resistant, then one of these antibiotics was incorporated in the plate to counterselect the donor, and a nutrient medium could be employed.

This method has the advantage of simplicity and is very useful if it is required to test large numbers of R^+ clones for transmissibility. Also, because of the high cell concentrations on the plate, transfer is detectable even if it takes place at very low frequency. The method is, however, entirely non-quantitative and measurements of transfer frequency etc. have to be obtained from broth matings, as described in (b).

(b) Broth matings. A broth culture of the R^- recipient was grown with aeration to a cell titre of 10^9/ml. while the R^+ donor was grown without aeration to 10^8 cells/ml. Equal volumes of the two cultures were mixed and allowed to stand at 37°C for a timed period before vortexing, diluting and plating on medium selective for the R^+ recipient.

Tests for surface exclusion (superinfection inhibition)

A broth mating was performed as described in section (b). The recipient strain contains the plasmid of unknown exclusion properties. Controls consist of an R^- (Sex^-) recipient and a recipient containing a plasmid known to exclude the donor (Sex^+ control). The index of exclusion was the ratio of:

$$\frac{\text{number of transfers to the } R^- \text{ recipient}}{\text{number of transfers to the } R^+ \text{ recipient}}$$

Tests for incompatibility

This was to discover if two plasmids could stably co-exist in the same cell. Each plasmid must have a phenotype which allows it to be detected in the presence of the other (usually this means they have distinguishable drug resistance patterns). Both plasmids were separately checked for their stable inheritance in the absence of drug selection. Transfers were then done by plate matings (as described above) in both directions (i.e. plasmid A → plasmid B and vice versa) and ten of the resulting colonies purified, maintaining selection only for the incoming plasmid. Purified colonies were streaked onto antibiotic ditch plates testing for the presence of both plasmids. If the resident plasmid was eliminated in all ten cases in both directions, the plasmids were incompatible.

Clones apparently harbouring both plasmids were further examined for stability by growing in drug-free medium for several generations, purifying, and retesting ten colonies for antibiotic resistance. Failure of the plasmids to co-exist in drug-free medium indicated incompatibility.

Clones stably inheriting antibiotic resistances from both plasmids were tested for their ability to transfer separately the drug resistance patterns of these plasmids. This was done by plate matings with appropriate antibiotic selection and ten transconjugants from both selections were purified. If all twenty had the resistances of both plasmids, recombination was considered to have occurred and the plasmids were incompatible.

Plasmid pairs which do not mutually eliminate each other and which are stably and separately inherited in the absence of antibiotic selec-

tion are compatible.

In cases where one or both of the plasmids to be tested were non-conjugative, then they were transferred by a mobilising plasmid known to be compatible with both of the test plasmids.

Isolation of *thy* strains

This was as described by Barth and Grinter (1974). 0.1 ml of a late log phase culture (5-10 x 10^8 cells/ml) was plated onto minimal medium containing the nutritional requirements of the strain together with 400 µM thymine and 10 µg/ml trimethoprim. Colonies obtained after 48 hours incubation were purified on the same medium before repeating the selection on medium containing trimethoprim and only 20 µM thymine to isolate low-thymine requirers (Barth *et al.*, 1968).

Isolation of *recA* strains

MA1079 is an Hfr *recA* strain which transfers the *recA* gene as an early marker, gene transfer being in an anticlockwise direction. The *thy* gene is close to *recA*. MA1079 was crossed with a *thy* recipient, selection being for Thy^+ in the recipient, and 5-10% of the Thy^+ transconjugants were found to have co-inherited the *recA* locus. Transconjugants were tested for U.V. sensitivity by streaking across a nutrient plate and exposing the open plate to the radiation from a Transilluminator Screen (U.V. Products Inc.) at a distance of 50 cm. The streaks were progressively covered by a card being moved over the plate, taking about 30 seconds to completely cover it. *recA* strains were killed by 2-5 seconds exposure, while $recA^+$ strains were not appreciably harmed by the full dose.

Growth and use of bacteriophage MS2 (Davis *et al.*, 1961)

Two 50 ml broth cultures of F^+ *E. coli* K12 were grown at 37° with aeration to a cell density of 3-5 x 10^8. About 10^7 p.f.u. of a stock of MS2 were added to one culture and both returned to shake for 2-3 hours

by which time the phage-infected cells have visibly lysed. Chloroform was added to this culture and it was shaken vigorously before spinning down the cell debris at 5000 x g for 10 minutes. The supernatant was serially diluted in broth and mixed with an F^+ tester strain before adding soft agar and pouring onto nutrient plates. A plaque count gave an estimate of the titre of the phage preparation. When testing for the presence of F pili on an unknown, a similar method was used, 10-100 p.f.u. of MS2 being added per plate. F^+ and F^- controls were included.

P1 transduction

The method adopted is basically as described in Barth *et al.* (1976). Transducing lysates were prepared by growing the donor bacterial strain in nutrient broth with aeration at 37^{o} to a titre of 5×10^8 cells/ml. 1M $CaCl_2$ was added to a concentration of 10mM and 0.25 ml samples of this culture added to 0.1 ml of a suspension of P1*kc* containing 5×10^7 plaque forming units (p.f.u.) per ml in P1 adsorption medium (this phage had been grown on an R^- λ^- bacterial strain). Adsorption was for about 25 minutes at $37^{o}C$. Soft agar was prepared by adding an equal volume of 20mM $CaCl_2$ to molten minimal agar (no salts) and cooling to around $40^{o}C$. 2.5 ml samples were added to the cells + phage and the mixture poured onto the surface of prewarmed, undried plates containing 50 ml of nutrient agar with 10mM $CaCl_2$. They were immediately incubated at $37^{o}C$ for roughly 16 hours. The top layers were removed with a glass spreader into sterile 50 ml centrifuge tubes, 0.25 ml of chloroform added and the mixture vortexed for about 30 seconds. The tubes were spun at $4^{o}C$ for 10 minutes at 12000 x g and the supernatants carefully removed with a pasteur pipette. The liquid was diluted 10-fold in P1 buffer and the dilution checked for sterility by plating 0.1 ml samples onto nutrient agar plates and incubating overnight.

Phage titres were assayed by mixing 0.1 mls of serial dilutions in

P1 adsorption medium of the phage preparation with 0.25 mls of P1 sensitive indicator (usually J53) grown as above, allowing adsorption, adding 2.5 ml soft agar and pouring onto ordinary dried nutrient plates which had been prewarmed. The plates were left on the bench 1-3 minutes until the soft layers were beginning to set and then incubated overnight. Phage plaques were counted and the titre was typically 5×10^8 to 5×10^9 p.f.u. per ml after the dilution in P1 buffer.

The transductional recipient strain was grown in 10 ml $CaCl_2$ broth[1] as above, centrifuged and resuspended in 1 ml of donor phage suspension (in P1 adsorption medium) at a multiplicity of infection (m.o.i.) generally between 0.01 and 0.1. After adsorption, sodium citrate solution was added to a concentration of 10 mg/ml and the cells centrifuged again and resuspended in 2.5 ml of nutrient broth containing sodium citrate (10 mgm/ml) before incubation for one hour at 37°C and plating onto suitable selective media containing sodium citrate (10 mgm/ml).[2]

Tris minimal medium (Hershey, 1955)

Per litre:				
Glucose	2.0 g (sterilised separately)	Tris	12.1 g
NaCl	5.4 g	KH_2PO_4	0.087 g
KCl	3.0 g	Na_2SO_4	0.023 g
NH_4Cl	1.1 g	$FeCl_3.6H_2O$	0.00027 g
$CaCl_2$	0.011 g	$MgCl_2$	0.095 g

P1 adsorption medium

10 ml Tryptone broth
1 ml 1M $CaCl_2$
89 ml sterile distilled water

---

1 When the temperature sensitive strain CRT46 was used, it was grown at 30°C with aeration in Hershey medium containing 10mM $CaCl_2$. All subsequent stages were carried out at 30°C.

2 The presence of sodium citrate was found to block the expression of tetracycline resistance by most plasmids [except those of incompatibility group N (N.J.G. unpublished)] and so it was omitted from plates containing tetracycline.

P1 buffer (Walker and Anderson, 1970)

20 mM Tris
10 mM $MgSO_4$ pH to 7.2 with dilute HCl
1% NH_4CH_3COO

Preparation of bacteriophage λ

A 10 ml broth culture of J62 was grown with aeration to a titre of about 2 x 10^8 cells/ml. The culture was poured into a sterile petri dish and irradiated at a distance of 15 cm from a U.V. Transilluminator Screen (U.V. Products, Inc., San Gabriel, California, USA) for 30 seconds. The culture was swirled continuously during irradiation. It was then returned to 37°C with aeration until the culture had cleared (60 to 90 minutes). 0.25 ml of chloroform was added and the cellular debris pelleted by centrifugation at 5000 x g for 10 minutes. The supernatant was carefully collected using a pasteur pipette. Plaque assays showed the preparation to contain between 10^9 and 10^{10} λ p.f.u. per ml.

Tests for λ lysogeny

Approximately 0.05 ml of a λ suspension prepared as above was streaked in a thin line across a nutrient plate using a loop. A parallel streak of λgv (a gift from R. W. Hedges) was made on the same plate. Cultures or colonies to be tested were streaked through the λ and λgv. λ^S, λ^{imm} and λ^R controls were included. Immunity to λ and sensitivity to λgv was taken to indicate lysogeny.

Preparation of λ DNA

1. Induction of a λ lysogen. This was either by thymine starvation or thermal induction. a) Thymine starvation (Korn and Weissbach, 1962). W3110T$^-$(λ) was grown at 37°C in 100 mls nutrient broth with aeration to a titre of 5 x 10^8 cells/ml. The cells were spun down at 10,000 x g for 10 minutes, the supernatant discarded, and the pellet resuspended in 100 mls M9 containing glucose (0.2%) and casamino acids (0.5%), but no thymine. The culture was shaken at 37°C for a further

$2\frac{1}{2}$ hours when thymine was added to a concentration of 10 μM. Shaking was continued for 2 more hours and then 0.5 mls of chloroform added. Vigorous shaking for several minutes caused the culture to lyse.

b) Thermal induction (Michaelis *et al.*, 1969). C600(λr14) was grown at 30° with aeration in 100 mls of M9 medium containing glucose (0.2%) and casamino acids (0.5%) for approximately 4 hours to a titre of 5×10^8. The culture was transferred to a water bath at 44°C for 20 minutes with intermittent swirling and then shaken at 37°C for 2 to 3 hours until the culture visibly lysed. 0.5 ml of chloroform was added and the culture shaken vigorously for several minutes.

2. Purification and concentration of λ phage particles (Wu *et al.*, 1972; Yamamoto and Alberts, 1970). The crude phage lysate from a) or b) above was spun at 10,000 x g for 10 minutes at 5°C and the supernatant collected. Bacterial DNA was digested by the addition of bovine pancreatic DNase (Sigma Chemicals, London) to a concentration of 1 μg/ml and $MgCl_2$ to 250 μM. After 20 minutes at room temperature, NaCl was added to 0.5 M with shaking until dissolved. PEG 6000 (B.D.H.) was added to 10% w/v and stirred at 10°C until dissolved. This caused a partitioning effect and the bacteriophage sedimented out of the mixture on standing overnight. Alternatively, it could be spun out at 10,000 x g for 10 minutes at 5°C. The pellet was resuspended in 1 ml of TES and the resuspension added to a 5 ml centrifuge tube. This was underlaid (using a syringe) with CsCl solutions in TES of progressively increasing density. Firstly 1 ml at 1.3 gm/ml, followed by 2 mls at 1.5 gm/ml and finally 1 ml at 1.7 gm/ml. The tube was topped with 0.2 mls of liquid paraffin and spun in a swing out rotor at 125,000 x g for 24 hours at 5°C. λ forms an opaque blue-white band between the 1.5 and 1.7 gm/ml layers and could be extracted using a syringe from above. The volume extracted was approximately 1 ml.

3. Extraction of λ DNA from phage particles (Bøvre and Szybalski, 1971). 1 ml of purified λ was dialysed at 5°C against four changes of 10mM phosphate buffer, pH 7.4. 10 µl of 25% w/v sodium dodecyl sulphate was added and the mixture heated to 60°C for 3 minutes to release λ DNA from the phage heads. The tube was cooled in ice and treated with an equal volume of redistilled phenol equilibrated with 0.1 M phosphate buffer (pH 7.4). The tube was gently inverted at room temperature for 1 minute and then spun at 5000 x g for 10 minutes at 5°C. The lower (phenol) layer was pipetted off and discarded and the extraction repeated twice more before dialysing the DNA against a continuous flow of 10 litres of TNE at 5°C.

DNA concentration was estimated by measuring the absorbance at 260nm using the relationship: 1 µg/ml of DNA ≡ absorbance of 0.024. Concentrations of between 200 and 500 µg/ml were obtained.

Isolation of labelled plasmid DNA

This was basically the method described in Barth and Grinter (1974). For *thy* strains the labelling medium was M9 supplemented with 0.2% glucose, 0.5% casein hydrolysate (Oxoid) and 6.6 µCi/ml ^{3}H thymine or 1.2 µCi/ml ^{14}C thymine (both from Amersham), and cold thymine to make the total thymine concentration 20 µM. For *thy*$^{+}$ strains, the cold thymine was omitted and deoxyadenosine added to 1mM. For strain J62, tryptophan (20 µg/ml) was also added.

Overnight standing cultures in labelling medium were diluted tenfold into 7.5 ml of fresh, prewarmed medium and grown with aeration at 37°C until they reached a titre of about 10^{9} cells/ml. This was usually after 4-5 hours. The cultures were cooled in ice and lysed by the method of Bazaral and Helinski (1968). Cells were sedimented by centrifugation at 0°C for 5 minutes at 5000 x g in an MSE Coolspin centrifuge. Pellets were resuspended in the same volume of cold TES

buffer and spun again. The cells were vortexed and then gently mixed with 0.5 ml of a solution of lysozyme (1 mg/ml), sucrose (100 mgm/ml) and RNase (0.5 mg/ml) in TES. After 20 minutes at room temperature the cells were lysed by the addition of 0.25 ml of sarkosyl (24 mg/ml) in TES. A further 0.5 ml of TES was added and the chromosomal DNA sheared by vortexing for 3 minutes before transferring the lysate to 10 ml polycarbonate centrifuge tubes. The volume was made up to 1.6 ml by the addition of TES followed by 5 mls of a TES solution containing 5.6 gms of caesium chloride (made by adding 112 g of caesium chloride to 69 mls TES) and 1 ml of a solution of 1.5 mg/ml ethidium bromide in TES. The tube's contents were mixed by inverting several times, topped with 1 ml of liquid paraffin and centrifuged at 200,000 x g for 65 hours at 20°C in a 10 x 10 titanium angle rotor in an MSE Superspeed 65 centrifuge.

Fractions (0.1 ml) were collected from a hole pierced in the bottom of the tubes using a collection apparatus essentially as described by Tan(1972). 5 µl samples were transferred to glass fibre discs (Whatman GF/C) which were dried and washed (about 50 at a time in 3 layers on filter papers in a Buchner funnel) in 400 ml of cold 5% trichloroacetic acid followed by 100 ml of acetone. The papers were dried again and their radioactivity counted in 5 mls of scintillant (7 gms/litre of 2-[4'-t-butyl-phenyl]-5-[4"-biphenylyl]-1,3,4-oxadiazole per litre of toluene) in an Intertechnique SL4000 liquid scintillation spectrometer.

Fractions containing supercoiled DNA shown by a peak in radioactive counts were pooled, the ethidium bromide removed by two extractions with equal volumes of isopropanol and then dialysed for 36 hours against a continuous flow of TNE buffer at 10°C. Plasmid DNA was stored at -20°C.

If the plasmid DNA was not to be used for sucrose gradient analysis, high activity was not necessary and the amount of label could be reduced by a factor of 5 and still allow a plasmid peak to be detected.

Transformation

The method adopted was a modification of that of Jacob and Hobbs, published in Barth and Grinter (1974). Recipient cells were *E. coli* C, *E. coli* C*thy* or ED5654 and the DNA was prepared as described above.

A standing overnight culture of the recipient in nutrient broth was diluted 1 in 20 into fresh, prewarmed broth and grown with aeration at 37°C for 100 minutes (Humphreys, 1978). The cells were chilled in ice and sedimented at 5000 x g for 5 minutes at 0°C in an MSE Coolspin centrifuge. Resuspension in the same volume of ice-cold 0.1 M $CaCl_2$ was followed by a second spin and resuspension in 0.5 volume of cold $CaCl_2$. The cells were left 20 minutes in ice before a third spin and resuspension in 0.05 volume of cold $CaCl_2$. The cells were now competent and 0.2 ml were mixed with 0.1 ml ice-cold DNA in TNE and heat shocked in a 37°C water bath for 1 minute with constant shaking before returning to ice for 1 hour. 2.7 ml prewarmed nutrient broth was added and the cells incubated at 37°C for another hour. Platings were made on nutrient agar with selective concentrations of an appropriate antibiotic.

Sucrose gradient sedimentation analysis of radioactively labelled plasmid DNA (Barth and Grinter, 1974 and 1977)

5 ml cellulose nitrate tubes (Beckman) containing 4.6 ml of a 12.5% solution of sucrose in TES were frozen at -70°C. The tubes were thawed for 100 minutes in centrifuge buckets held in a block of Styrofoam and the gradients formed approximated very closely to theoretical isokinetic 5-20% gradients (P. T. Barth, personal communication).

Intact or restriction enzyme digested ^3H labelled plasmid DNA was

carefully loaded onto the top of the gradient together with a suitable ^{14}C labelled DNA to act as a molecular weight marker. The total volume added did not exceed 0.2 ml. Tubes were spun at 100,000 x g in a 6 x 5.5 ml swing out aluminium rotor at 20°C in an MSE Superspeed 65 centrifuge for a time which depended on the molecular weight of the DNA being analysed.

0.1 ml fractions were collected onto Whatman GF/C filters which were washed, dried and counted as described above in "Isolation of labelled plasmid DNA". The exact volume of the gradient was noted.

Molecular weights (M) were calculated from the ratio of sedimentation distances (d) of DNA molecules with similar tertiary structures, using the equation:

$$\left[\frac{M(\text{species A})}{M(\text{species B})}\right]^{e} = \frac{dA}{dB}$$

The sedimentation exponent (e) was empirically derived as 0.34, 0.35 and 0.42 for linear (LIN), open circular (OC) and supercoiled (CCC) forms respectively (P. T. Barth, personal communication). The ratio of the distances sedimented by the various tertiary structures of a DNA molecule were given by the relationships:

$$\frac{dA(CCC)}{dA(OC)} = 1.20 \times M_A^{0.07}$$

and

$$\frac{dA(OC)}{dA(LIN)} = 1.09 \times M_A^{0.01}$$

Restriction enzyme digestions

All digestions were done in 2 ml Eppendorf conical polypropylene tubes. To the DNA in TNE was added 0.1 volume of appropriate buffer (see "Buffers" above) and 0.01 volume of the enzyme (Miles Ltd.). Digestion was for 60 minutes at 37°C. Residual enzyme activity was destroyed by heating to 60°C for 10 minutes.

DNA ligations

Restricted DNA prepared as above was dialysed overnight against a continuous flow of 5 litres of TNE. 0.01 volumes of 5 mM ATP, 1.5 M $MgCl_2$ and DNA ligase (Miles Ltd.) were added and the mixture left overnight at 10°C. It was dialysed again and then used for transformation as already described.

Gel electrophoresis

a) Vertical tube gels. As described in Crinter and Barth (1976). See end of thesis.

b) Vertical slab gels. 0.7 or 0.8% agarose (Sigma Chemical Co.) was made up in borate buffer. The gel dimensions were 15 x 10 x 0.5 cm. Because of the large well-size, these gels were particularly suitable for the single colony lysis technique for which they were used exclusively.

The method was adapted from Eckhardt (1978) by P. L. Bergquist (personal communication). 25 µl of a lysozyme mixture was added to each well. This mixture contained lysozyme (7500 u/ml), RNase (0.3 u/ml pretreated at 80°C for 10 minutes in 400 mM sodium acetate buffer, pH4), 0.05% w/v bromophenol blue and 20% w/v ficoll (molecular wt. 400,000) all made up in tris-borate electrophoresis buffer. Using a toothpick, a large single colony from a fresh nutrient plate was smeared against the glass at the side of a well and washed down into the well with the lysozyme mix. This avoided the formation of lumps. After

5 minutes, 35 µl of 10% w/v ficoll containing 0.2% w/v SDS in electrophoresis buffer was added and the two layers mixed (very gently to avoid shearing) with 2 or 3 strokes of a toothpick. Complete mixing was not essential. Finally 50 µl of 5% w/v ficoll containing 0.2% SDS in borate buffer was added carefully down the side of the well to form an upper layer. The wells were capped with the same agarose as used to make the gel. Electrophoresis was for 3-6 hours at 130V. The gel was stained in borate buffer containing 5 µg/ml of ethidium bromide for 10 minutes, thoroughly washed in running distilled water; wiped with a tissue and viewed on a Transilluminator Screen (UV Products Inc.). Photographs were taken on Poloroid type 107C film through a red filter. The gels were calibrated by the inclusion of at least three plasmids of known molecular weight to act as standards.

c) Horizontal slab gels. The gel dimensions were 14 x 14 x 0.5 cm (McDonell *et al.*, 1977). All horizontal gels were run with restriction enzyme digested plasmid DNA, prepared as described above, made up to a final volume of 50 µl with TNE. To this was added glycerol, bromophenol blue and 1% agarose to concentrations of 5, 0.1 and 0.25% respectively. The samples were mixed, placed in the wells and capped with agarose. Ethidium bromide (1 µg/ml) was included in the gel. Electrophoresis was at 150 V for 2½-6 hours, depending on the size of fragments being resolved. Gels were photographed as above. Calibration was with the fragments obtained by *Eco*RI and *Bgl*II single and double digests of λ DNA (Thomas and Davis, 1975; Kamp *et al.*, 1977). Analytical gels were of 0.7 or 0.8% agarose (Sigma Chemical Co.) in borate buffer. Preparative gels were of 1% Sea Plaque agarose (Marine Colloids Inc.) in acetate buffer and DNA restriction fragments were extracted from them as described below.

Extraction of DNA from gels (Langridge, Langridge and Bergquist,

manuscript in preparation)..

A two phase solution was prepared as described in Hurst and Sheng (1976). 1-butanol (150 ml) and glass distilled water (150 ml) were equilibrated by shaking in a separating funnel. The two phases were separated and 1 gm cetyltrimethylammonium bromide (B.D.H.) was dissolved in the butanol fraction which was shaken with 100 ml of the aqueous fraction. 50 µl of Antifoam A (Sigma Chemical Co.) was added. The solution was left overnight for the phases to separate, when each was bottled separately and stored at 37°C.

A horizontal gel was prepared and run as described above, using 1% Sea Plaque agarose (Marine Colloids Inc.) in acetate buffer containing 1 µg/ml ethidium bromide. After electrophoresis, the gel was viewed on a Transilluminator Screen (U.V. Products Inc.) and the desired bands cut out with a scalpel and transferred to a polypropylene tube. The extract was melted at 70°C and cooled to 37°C. Equal volumes of aqueous and butanol phases were added, the tube inverted about 50 times and then left at 37°C for the phases to separate. The butanol layer was removed and kept, the extraction repeated twice more and the extracts combined. 0.25 volume of 0.2 M NaCl was added to the extract and the tube inverted about 50 times. The lower aqueous layer was removed and kept, the extraction repeated and the extracts combined. An equal volume of chloroform was added dropwise and the solution placed in ice for the cetyltrimethylammonium bromide to precipitate. The chloroform was removed, the residual evaporated off in a stream of air and the DNA dialysed for 36 hours at 10°C against a continuous flow of 5 l. of TNE. During the extraction, ethidium bromide is removed to the butanol layer, where it remains.

The DNA fragments isolated in this way can now be ligated into suitable cloning vehicles.

Table 1.1. Bacterial Strains: *Escherichia coli* K12

Strain	Characteristics	Reference or source
J53	F⁻ *proA metF* $(\lambda)_R$	Clowes and Hayes (1968)
J53-1	F⁻ *proA metF* Nal^R (λ)	" "
J53-2	F⁻ *proA metF* Rif^R (λ)	" "
J62	F⁻ *lac his trp proC* $(\lambda)_R$	" "
J62-1	F⁻ *lac his trp proC* Nal^R (λ)	" "
J62-2	F⁻ *lac his trp proC* Rif^R (λ)	" "
C600	F⁻ *lacY1 thr leu thi supE44*	" "
C600*recA*(HH26)	F⁻ *lacY1 thr leu thi supE44 recA*	Barth *et al.* (1976)
W3110T⁻	F⁻ *thyA dra*	Bachmann (1972)
W3110*recA*(HH27)	F⁻ *dra recA*	Barth and Datta (1977)
W3110T⁻-Tn7 (formerly W3110T⁻-TnC)	F⁻ *thy dra* chromosomally inserted Tn7	Barth and Grinter (1977)
AB1157	F⁻ *thr leu thi proA his argE str*	Bachmann (1972)
AB1450	F⁻ *argH his metB ilvD str*	"
CRT46	F⁻ *thr leu thi ilv thi dnaA*(ts)	Hirota *et al.* (1968) [Received from L. Caro, Geneva]
MA1079	Hfr *recA ser thi*	Received from W. Maas
ATCC14948*thy*	F⁻ *thy*	Barth and Grinter (1974)
ED5654	F⁻ *lac met* r^- m^+	Received from N. Murray, University of Edinburgh
W3110*trpA33*Rif	*trpA33* Rif^R	Barbara Bachmann
W3110*trpB*	*trpB*	" "
W3110*trpC*	*trpC*	" "
W3110*trpD*	*trpD*	" "
W3110*trpE5*Rif	*trpE5* Rif^R	" "
4100	*thi strA argF*	Nicolas Glansdorff
30S0	Hfr *argG* Nal^R	" "

Bacterial Strains other than *E. coli* K12

E. coli C	F⁻ *lac met* r^- m^-	Wiman *et al.* (1970)
E. coli C*thy*	F⁻ *lac met thy* r^- m^-	Grinter and Barth (1976)

Table 1.2. Bacterial Plasmids

Plasmid	Molecular mass (Mdal)	Phenotype	Reference
R300B	5.7	$Sm_R^R Su_R^R$	Barth and Grinter (1974)
R305C	5.7	$Sm_R^R Su_R^R$	" "
R310	5.7	$Sm_R^R Su_R^R$	" "
R450B	5.7	$Sm_R^R Su_R^R$	" "
R464C	5.7	$Sm_R^R Su_R^R$	" "
R676	5.7	$Sm_R^R Su_R^R$	" "
R678	9.2	$Sm_R^R Su_R^R$	" "
R682	5.7	$Sm_R^R Su_R^R$	" "
R684	6.3	$Sm_R^R Su_R^R$	" "
R750	5.9	$Sm_R^R Su_R^R$	" "
R1162	5.5	$Sm_R^R Su_R^R$	" "
PB165	7.4,14.7,21.4	$Sm_R^R Su_R^R$	" "
R7K-TnC1	33.5	$Ap_R^R Tp_R^R Sm^R Tra^+$	Barth *et al.* (1976)
Ap201	9.1	$Ap_R^R Sm_R^R$	Heffron *et al.* (1975)
R144drd3	65	$Tc_R^R Km_R^R Tra^+$ (derepressed)	Meynell and Datta (1967)
RP4	36	$Ap_R^R Tc_R^R Km^R Tra^+$	Datta *et al.* (1971)
RP4δ1	28	$Ap_R^R Tc_R^R Tra^- Sex^-$	Hedges *et al.* (1976)
R702	46	$Km_R^R Sm_R^R Su^R Tc_R^R Hg_R^R Tra^+$	Hedges and Jacob (1974)
R1033	45	$Ap^R Tc^R Km^R Cm^R Gm^R Su^R Tra^+$	Smith *et al.* (1975)
pRP1 (formerly RP4-TnC1)	45	$Ap^R Tc^R Km^R Tp^R Sm^R Tra^+$	Barth and Grinter (1977)
R68.44	36	$Ap_R^R Tc_R^R Km_R^R Tra^+$	Jacob *et al.* (1976)
RA1-1	90	$Cm_R^R Tc_R^R Su_R^R Tra^+$	Datta and Hedges (1973)
R40a	96	$Ap_R^R Km_R^R Su_R^R Tra^+$	Chabbert *et al.* (1972)
R1	62	$Ap^R Cm^R Km^R Sm^R Su^R Tra^+$	Meynell and Datta (1966)

Table 1.3. Bacteriophages

Phage	Molecular mass (Mdal)	Reference or source
λ	30.8	Hershey (1971)
λr14	31.3	Hirsch *et al.* (1972)
P1*kc*	59	Hedges *et al.* (1975)
ØAmp	65	"
P1CM	60	"
MS2	-	Davis *et al.* (1961)

CHAPTER 2

THE SPREAD OF PLASMID-BORNE ANTIBIOTIC RESISTANCE

Abstract

Plasmids conferring linked resistance to streptomycin and sulphonamide were compared in their susceptibility to cutting by restriction enzyme and by incompatibility testing. The plasmids came from several different hosts in widely separated locations and had already been shown to be of similar molecular weight and to possess a high degree of DNA sequence homology. The further comparisons confirmed the close relatedness of this group of plasmids, suggesting that they represented one plasmid clone that had spread very widely in response to selective pressure. One plasmid, PB165, was shown to form oligomers in *E. coli* and this allowed models for control of plasmid replication to be tested.

Introduction

Whereas plasmid transfer can be readily demonstrated in the laboratory, it is apparent that transfer *in vivo* occurs relatively rarely. Factors such as temperature, pH, Eh, availability of nutrients, population density, presence of bacteriophages and chemical inhibitors and absence of a positive selection in the environment for the products of transfer, all reduce the number of transfers detected.

A number of studies have established that transfer of antibiotic resistance plasmids can occur in the alimentary tract of man and animals (Jarolmen and Kemp, 1969; Reed *et al.*, 1969; Smith, 1971; Walton, 1966); in most of these studies the normal flora of the alimentary tract was modified by antibiotic treatment or by using germ-free experimental animals. Transfer in a host with normal gut flora was a rare event. One subject (Smith, 1969) swallowed overnight broth cultures of *E. coli* harbouring R-factors at regular intervals over a two year period. Transfer was monitored to the serologically defined, nalidixic acid resistant *E. coli* which was the predominant species resident in his gut. Transfer was detected, but at a very low level. There was no evidence for an epidemic spread of the plasmids to the resident *E. coli*, nor was there persistence in the gut of R+ residents.

The rarity of plasmid transfer seems largely due to the normal gut flora, particularly *Bacteroides* spp., whose presence and metabolic activities create conditions hostile to colonisation by and transfer from an invading R^+ strain. Consequently, pre-treatment with antibiotics which may remove the resident flora will give a newly introduced plasmid-containing strain a better chance of becoming established and of transferring its R-factor (Anderson, 1975). Meynell (1963) showed that in mice a single dose of streptomycin modified the pH, Eh

and concentration of volatile fatty acids in the gut. Simultation of these changes *in vitro* gave roughly a 100-fold increase in plasmid transfer (Falkow, 1975). Therefore, antibiotics not only select for strains carrying R-factors, but also give rise to conditions which favour their transfer by conjugation.

The extent if not the frequency of R-plasmid transfer *in vivo* can be judged from epidemiological studies. If resistance to particular antibiotics in widely separate geographical locations is due to the same or very closely related plasmids, this suggests efficient spread of an ancestral plasmid. Sulphonamide (Su) and streptomycin (Sm) were the first effective antibacterial drugs to be used therapeutically, and plasmids coding for linked resistance to SmSu are very common (Anderson and Lewis, 1965; Bryan *et al.*, 1972; Hedges *et al.*, 1973; Lawn *et al.*, 1967; Skovgaard and Nielsen, 1972; Smith and Linggood, 1970). Smith *et al.*, 1974, examined 26 such plasmids and found that 19 were of one compatibility group. Barth and Grinter (1974) compared twelve SmSu plasmids from a variety of host strains from widely separated locations (Table 2.1). Ten of the twelve were indistinguishable in terms of their molecular weight and DNA homologies. The remaining two were somewhat larger, but still showed appreciable homology. Smith *et al.* (1974) suggested that similar selective conditions - i.e. the use of antibiotics therapeutically - in different parts of the world had led to the parallel evolution of similar plasmids,but the very close relationship between most of the SmSu plasmids described by Barth and Grinter, 1974, makes a common origin for them seem more probable.

This chapter is concerned with the further characterisation of the same SmSu plasmids (Table 2.1). It was hoped to detect small differences in their DNA sequences by comparing their sensitivity to the restriction enzyme *Eco*RI, and by testing their incompatibility

Table 2.1. Origins and properties of 12 SmSu resistance plasmids (Data from Barth and Grinter, 1974)

SmSu resistance plasmid	Origin: Bacterial species	Origin: Geographical location	Molecular mass (Mdal)	% age homology with R300B reference[1]	No. of copies per chromosome
R300B	*S. typhimurium*	U.K.	5.7	100	10.9
R305C	"	U.K.	5.7	84.2 ± 11.9	9.3
R310	"	U.K.	5.7	84.5 ± 11.2	9.2
R450B	*P. morganii*	S. Africa	5.7	91.9 ± 4.4	8.6
R464C	"	"	5.7	82.4 ± 6.0	11.2
R676	*S. senftenberg*	Denmark	5.7	79.4 ± 8.7	8.5
R678	*S. dublin*	"	9.2	45.7 ± 4.8	10.8
R682	*P. mirabilis*	Germany	5.7	92.5 ± 7.5	7.9
R684	"	"	6.3	83.9 ± 8.0	10.5
R750	*Providencia* sp.	U.S.A.	5.9	85.6 ± 13.7	11.5
R1162	*Pseudomonas aeruginosa*	Canada	5.5	93.0 ± 5.9	11.6
PB165	*E. coli*	U.K.	7.4,14.7,21.4[2]	35.2 ± 5.3	4.1,1.3,0.8[3]

Table 2.1 Continued.

Origins and properties of 12 SmSu resistance plasmids (Data from Barth and Grinter, 1974)

1 R300B homoduplex formation varied from 81-92%. All heteroduplex results are expressed relative to this control, set to 100%. Homology was assayed using a single-stand specific nuclease which digests any unpaired DNA sequences (Barth and Grinter, 1975).

2 PB165 was found to consist of at least 3 inseparable plasmid species. Their molecular weights are consistent with their being oligomerically related to a 7.4 Md monomer.

3 These are the respective copy numbers of the three plasmid species based on their relative preponderance as measured by sucrose gradient analysis.

properties. The results confirmed the close relationship of these plasmids. All belonged to one incompatibility group. Ten had a single *Eco*R1 cut site. PB165 (Table 2.1), on *Eco*R1 cleavage, yielded linear molecules of molecular weight 7.6×10^6 only, confirming that the closed circular molecular species were monomers, dimers and oligomers. R678 (Table 2.1), whose M.wt was 9.2×10^6, higher than the others, had three *Eco*R1 cut sites, consistent with its being a sister-molecule of the others, but with an insert bearing two cut sites. These findings reinforced the view that the plasmids had a common ancestor rather than having evolved separately. The work was published (Grinter and Barth, 1976) and a copy is bound in with this thesis.

CHAPTER 3

ANALYSIS OF THE TRANSFER GENES ON A SELF-TRANSMISSIBLE PLASMID

Abstract

Mutagenesis by transposon 7 insertion was used to generate transfer deficient derivatives of RP4. The mutants were characterised according to:

1. Their transfer frequency
2. Their ability to prevent the entry of a related superinfecting plasmid
3. Their ability to complement each other.

This last property was tested using P1 transduction to create temporary heterozygotes between pairs of *tra* mutants and looking for restoration of transferability.

In addition, some *tra* mutants which were known to produce pili were tested for their ability to mobilise a small, non-conjugative plasmid.

The RP4 transfer system was compared and contrasted with that of F.

Introduction

Bacterial conjugation was first demonstrated by Lederberg and Tatum (1946). The requirement for cell to cell contact (Davis, 1950) distinguished this mode of gene transfer from other forms of bacterial genetic exchange like transduction and transformation. Chromosomal transfer mediated by the fertility factor F in *Escherichia coli* has been invaluable in genetic mapping (for a review, see Hayes, 1968 and this thesis, Chapter 5). Much work has also gone into understanding the genetics of F itself (for a review, see Manning and Achtman, 1979) and to date it is the only plasmid to have had its transfer system extensively characterised.

The F factor is a double stranded circular DNA molecule of molecular weight 63 Megadaltons (Jacob *et al.*, 1977a) which is sufficient to encode about 100 proteins. About one third of the molecule codes for functions relating to transfer and most of the genes involved form a single operon (Helmuth and Achtman, 1975). F-mediated conjugation is therefore likely to be a complex process.[1] A map of the F plasmid is shown in figure 3.1.

Genetic and physical analysis of conjugation involving transfer of the F factor has allowed a number of stages to be distinguished. This study has been aided by the isolation of mutations in F which affect the different steps and has led to the identification of more than twenty genes concerned with F transfer. The various stages and the genes involved are considered below in some detail.

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1 It seems generally true that bacterial mating involves many genes since even the smallest known conjugative plasmids (IncW) are larger than 20 Megadaltons (Jacob *et al.*, 1977a).

Figure 3.1: Genetic map of the F factor.

The distances shown around the molecule are percentages of the F genome. Abbreviations are: phi for phage inhibition; oriV for origin of vegetative replication; rep for F replication; inc for incompatibility; oriT for origin of transfer; finP for the F specific component of the FinOP repression system; tirO for the site of action of transfer inhibitors. Also shown are regions concerned with Hfr and F' formation. Data are from: Hu et al.,1975a; Sharp et al., 1972; Skurray et al., 1976; Thompson and Achtman, 1978; Willetts, 1972.

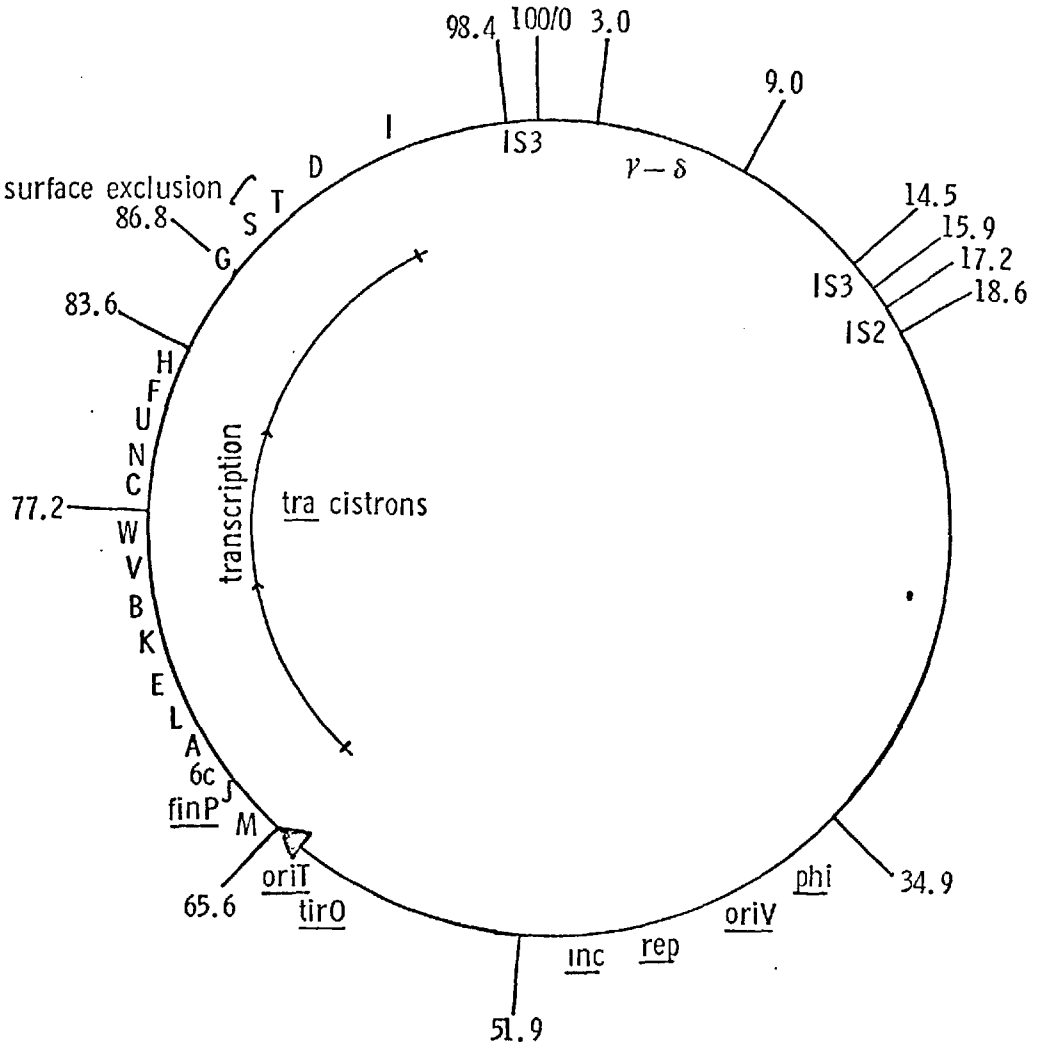


Figure 3.1 : Genetic map of the F factor .

Stages in F-mediated conjugation

1. F pilus binding and formation of wall to wall contacts. Virtually all F^+ cells produce special structures on their surface known as F pili. They can be recognised immunologically (Ørskov and Ørskov, 1960; Ishibashi, 1967) or by electron microscopy (Crawford and Gesteland, 1964). In order to distinguish these pili in the electron microscope from other similar structures on the cell surface (common pili and flagella) they can be labelled with male-specific phages which attach to receptor sites on the pili, or with pilus-specific antibody.

The role of F pili in conjugation is still not clearly understood. They are important in establishing the initial contacts between cells because their removal by high speed blending (Novotry *et al.*, 1969) or dissolving with SDS (Achtman *et al.*, 1978a) abolished conjugation by preventing the formation of mating aggregates until new pili could be synthesised. Bacteriophages which adsorb to the tips of F pili also block the formation of mating aggregates (Ou, 1973). Ou and Anderson, 1970, showed that wall to wall contact was not necessary for DNA transfer, suggesting that pili were a conduit for transferred DNA. This is probably not the normal route for DNA transfer, however, since mating aggregates are not disrupted by SDS, nor is DNA transfer prevented.

It is possible that F pili form the initial cell to cell connection and are then retracted, bringing the mating cells into close, wall to wall contact (Curtiss, 1969), but the evidence is not conclusive. Efforts to determine an F pilus receptor site using preparations of purified pili have been unsuccessful because of the high degree of non-specific binding observed (Helmuth and Achtman, 1978).

2. Stabilisation of mating contacts. Two genes, *traG* and *traN* (Figure 3.1), appear to be involved in the formation of stable mating associations. *traG* mutants can complement those in *traN*,

(Willetts and Achtman, 1972) but F^+ cells carrying either defect appear unable efficiently to form stable aggregations with recipients whether analysed by Coulter counter or electron microscopy (Achtman and Skurray, 1977).

In addition, the recipient plays an important role in this process. Apart from being F^- (see "Surface exclusion" below), it must have certain components in the cell surface which can be recognised by the donor. Many mutants defective in recipient ability are known, and a comparative study has been undertaken (Achtman *et al.*, 1978b). All were found to have alterations in the cell envelope. They were either mutants in outer membrane protein A (*ompA*), or showed altered lipopolysacchoride structure (L.P.S.). While these defective recipients gave normal levels of mating aggregation in liquid under conditions giving low shearing forces, transfer of DNA was reduced as much as 5000-fold. This effect was not seen when matings were carried out on membrane filters, suggesting the defect is in the ability to form stable mating contacts. The *ompA* product is specific for F transfer, since R100-1, an F-like plasmid with DNA sequence homology with F in the *tra* region (Cohen, 1977), transfers to an *ompA* mutant at normal frequency (Achtman and Skurray, 1977). The function of L.P.S. *in vivo* is probably an interaction with the *ompA* product, protecting it from proteolytic cleavage (Enderman *et al.*, 1978; Schweizer *et al.*, 1978).

3. Triggering of DNA replication. Kingsman and Willetts (1978) examined *dnaB*^{ts} mutants carrying *tra* mutants of F-*lac*. At the restrictive temperature, chromosome replication ceases, allowing the detection of F replication associated with transfer. F-*lac* mutants at two loci, *traI* and *traM* (Figure 3.1), were unable to initiate F replication when mixed with F^- recipients under these conditions even though they formed stable mating aggregates (Manning and Achtman, 1979). These obser-

vations suggest that *traI* and *traM* are responsible for the triggering of F DNA replication associated with transfer. *traG* and *traN* mutants which cannot form stable mating aggregates (see 2 above) are nevertheless still capable of triggering F replication, suggesting it occurs at an early stage in the mating cycle.

Manning and Achtman, 1979, suggested that *traI* and *traM* counteract transfer inhibitors which act at a site *tirO* (Figure 3.1) very close to the origin of transfer (*oriT*).

4. Transfer. Nothing is known about the enzymes involved or the cell components necessary for transfer. The only F coded proteins implicated in transfer are the *traD* product (Achtman and Skurray, 1977) and possibly the 6c (Figure 3.1) protein which is plasmid specific (Thompson and Achtman, 1978). Transfer is of a single strand, 5' end first (Rupp and Ihler, 1968), starting from *oriT* (Willetts, 1972). Whether transfer is of multiple copies of F by a rolling circle mechanism, or whether it is terminated after transfer of a single genome is not known (Achtman and Skurray, 1977). However, at least one single stranded copy is transferred in a few minutes at 37°C.

5. Disaggregation. Completion of at least one round of F DNA transfer triggers destabilisation of the mating aggregates. This process was essentially complete at 37°C within 30 minutes after DNA transfer was complete (Achtman *et al.*, 1978a). It was possible to identify disaggregation as a specific event because Hfr x F⁻ cells do not show this property and therefore it is not the result of spontaneous decay. The basis of the property is not understood, but may be linked with the initiation of pilus synthesis following completion of transfer.

6. F pilus synthesis. Purified F pili consist of a single detectable subunit of molecular weight 11,000 daltons (Helmuth and Achtman, 1978). However, at least 13 genes are involved in pilus

production, namely *traJ*, *A*, *L*, *E*, *K*, *B*, *V*, *W*, *C*, *U*, *F*, *H* and part of the *traG* gene (Figure 3.1). This suggests there are many genes involved in processing, assembly and control during pilus synthesis. *traJ* is also necessary for the expression of surface exclusion (see below). Since the other surface exclusion genes are not required for transfer, it was suggested that the *traJ* product was involved in the positive control of the *tra* operon (Finnegan and Willetts, 1973). Willetts, 1977, showed *traJ* was necessary for transcription of *tra* operon cistrons, confirming its regulatory role.

There was also good evidence that *traA* was the structural gene for F pilin. Male specific bacteriophages showed altered specificity for some *traA* mutants (Willetts, 1971). More convincingly, suppression of amber mutants in *traA* resulted in the production of an altered F pilin (Minkley *et al.*, 1976).

More recent evidence has pointed to different roles for *traA* and *J*. The *traA* product (and F pilin) were not detected in the cell envelope (Achtman *et al.*, 1977) whereas the *traJ* protein occasionally was (unpublished results quoted in Manning and Achtman, 1979). The *traJ* product was not required for transcription of the *tra* genes in mini cells or *in vitro* (Kennedy *et al.*, 1977). The *traA* protein synthesised *in vitro* was not precipitated by anti F pilus antiserum (Kennedy *et al.*, 1977) whereas the *traJ* product was preferentially precipitated (Helmuth, 1979). Moreover, when the tryptic fingerprints obtained from the *traJ* and *A* proteins were compared with that of F pilin only the *traJ* product showed similarity (Helmuth, 1979).

All this new evidence pointed to *traJ* being the structural gene for F pilin production. The molecular weight of its product is 23,000 daltons whether synthesised *in vitro* or isolated from the cell envelope so it must be processed before or during pilus assembly to

give the characteristic 11,000 dalton subunit. It may still have a regulatory role in transcription, or instead, another protein may recognise the unprocessed *traJ* product in the cell envelope and switch on the *tra* operon. Such a function may have been detected in *sfr* strains (Beutin, 1978) which are chromosomal mutants causing pleiotrophic effects in F expression.

The role of *traA* is now not certain, but it may be important in processing precursors of pilus protein.

Control of *tra* genes

In addition to genes directly involved in transfer, there are those affecting expression of the *tra* genes. *ompA*, L.P.S. and *sfr* mutants and the *tirO* site have already been mentioned. Other genes in this category are now considered.

Surface exclusion. This property is shown by F^+ cells which act as very poor recipients in conjugation with another F^+ donor. Apart from the need for the *traJ* product for expression of surface exclusion (see 6. above), two other genes *traS* and *traT* (Figure 3.1) are involved. These genes are not required for transfer, but cells carrying *traS* or *traT* mutants of F are better recipients than those with wild-type F (Achtman *et al.*, 1977).

The *traS* product appears to be plasmid specific whereas *traT* protein is produced by other F-like sex factors (but in lesser amounts). Cloning of the *traS* and *traT* region of F (Figure 3.1) showed the level of surface exclusion correlated with the concentration of the *traT* protein in the outer membrane. Isolation of *traS* or *traT* mutants of the cloned DNA showed the two cistrons were partially and independently responsible for surface exclusion and that they normally function co-operatively (Achtman *et al.*, 1977).

Conjugation between F^+ donors and *traT*$^+$ recipients closely resembles that between *traG* or *traN* F^+ donors and F^- recipients (see 2. above). This suggests that *traT* functions by preventing stable mating aggregation, possibly by masking an as yet unknown conjugation receptor in the outer membrane (Manning and Achtman, 1979).

traS protein is localised in the inner membrane (Achtman *et al.*, 1977) and where present in the recipient it does not prevent stable mating aggregation. Ideas about its mode of action stem from the work of Ou (1975) who crossed F^+ x F^+ cells and detected no F replication; in contrast with F^+ x F^- crosses. He then looked at crosses with F^+ donors and minicells as recipients. Minicells from F^+ and F^- strains both triggered F DNA synthesis, but transfer was detectable only to the minicells produced by an F^- strain. Since the *traT* protein is stable while that of *traS* is subject to rapid turnover (Achtman *et al.*, 1977) it seems likely that the *traS* product is inactive in minicells. The function of *traS* is therefore to prevent triggering of F replication in the donor. This triggering is also needed for DNA transfer since a *traS*$^+$ recipient prevents transfer within stable mating aggregates (Achtman *et al.*, 1977). Once triggered, replication and transfer can apparently proceed independently.

Repression of transfer - the *finP* cistron. The F factor is unusual in being derepressed for the expression of the transfer genes. Many other F-like sex factors are repressed, showing lower transfer frequencies and the ability to repress F transfer when present in the same cell (Meynell and Datta, 1966). In cultures carrying such plasmids, only a small minority of the cells bear sex pili. Repression in this system involves the *finO* product of the F-like plasmid which interacts with the *finP* product of F at a site, *fisO*, mapping very near *traJ* (Figure 3.1); turning off *traJ* transcription (Finnegan and

Willetts, 1973). The *traJ* protein is needed for transcription of the remainder of the *tra* operon (except *traM*, Willetts, 1977 and section 6 above) and F behaves like a *traJ* mutant in the presence of a repressing F-like plasmid. However, *finP* mutants of F are not affected by the presence of other F-like sex factors, expressing surface exclusion and giving normal levels of transfer. The F factor is naturally mutant at *finO* and so is normally derepressed for transfer even though it produces the plasmid-specific *finP* product.

Transfer systems of other plasmids

There is available very little information of the type presented above on the conjugation systems determined by other transmissible plasmids. Some sex factors share extensive DNA homology with F (Sharp *et al.*, 1973), while others are unrelated (Falkow *et al.*, 1974). Conjugation mediated by I group plasmids (Hedges and Datta, 1973) bears resemblances to that of F and F-like sex factors, including repression of the transfer function (Meynell and Datta, 1967) and production of a sex pilus which is specifically recognised by bacteriophages (Meynell and Lawn, 1968). Incompatibility groups P, N and W also encode characteristic pili and confer phage sensitivity (Bradley and Rutherford, 1975; Bradley and Cohen, 1977).

Another feature shared by many conjugative plasmids is specific surface exclusion, but this is not seen between W plasmids, the smallest known conjugative plasmids.

Plasmids of several incompatibility groups with transfer functions unrelated to F or I, determine the production of pili in all or a majority of the cells in a culture. This applies to plasmids of IncA, IncC, IncD, IncN, IncP, IncT and IncW (Bradley, 1979), in contrast to IncFII and IncIα, the pili of which are undetectable in normal culture

by electron microscopy.

Detailed knowledge of other plasmid transfer systems is virtually non-existent. The work presented in this chapter is an extension of that presented by Barth *et al.*, 1978, (a copy of which is bound at the end of this thesis) and represents the beginnings of a genetic analysis of the transfer system of the IncP plasmid RP4.

Results

Isolation of *tra* mutants. RP4 was mutagenised by the insertion of transposon 7 (Tn7) using the technique described by Barth and Grinter, 1977. Tn7 is a transposable DNA sequence of about 9 Md. carrying genes for resistance to trimethoprim and streptomycin (Barth *et al.*, 1976). Derivatives of strain W3110T$^-$ or J62 having a copy of Tn7 in the chromosome were infected with RP4. Such strains [known as W3110T$^-$-Tn7(RP4) or J62-Tn7(RP4)] were mated in liquid with J53-2 and plated on lysed blood medium containing trimethoprim (10 µg/ml) together with rifampicin (50 µg/ml) to prevent growth of the donor.

Trimethoprim resistance was transferred at a frequency of about 1×10^{-5} per donor per hour (see Chapter 4). Transconjugant clones were tested for antibiotic resistance pattern and their ability to transfer trimethoprim or tetracycline resistance to C600 by plate mating (Chapter 1). All trimethoprim resistant colonies had the unselected streptomycin resistance of Tn7. A very few clones (< 1%) inherited no RP4 markers and presumably were formed either by mobilisation of the chromosomal region in the donor carrying Tn7 (without RP4 itself transferring) or by transposition to RP4 into a site preventing its replication, this defective plasmid then being transferred and Tn7 re-transposed to the recipient chromosome. The rest of the clones carried some or all RP4 markers. All were ampicillin resistant, but about 2% had lost either tetracycline or kanamycin resistance. Of the remainder, about 2-3% had wholly or partly lost the ability to transfer their drug resistances to strain C600. Two clones were isolated which transferred RP4 markers at a normal frequency (1×10^{-3} per donor per hour) but which transferred trimethoprim resistance at a much lower frequency. It was concluded that these had inherited a normal RP4 and a copy of Tn7 in the chromosome.

Analysis of plasmid DNA from about 50 trimethoprim resistant transconjugants showed them to contain a single plasmid species of molecular weight consistent with their being an RP4 molecule (36 Md) containing a single copy of an inserted Tn7 (9 Md). Further, mapping the sites of insertion showed that those plasmids which were kanamycin or tetracycline sensitive had insertions which clustered at particular sites indicating that they were formed by inactivation of the drug resistance gene by insertion of Tn7 (Barth and Grinter, 1977). Insertions giving rise to *tra* mutants were found in two widely separated regions on either side of the kanamycin resistance gene (Barth *et al.*, 1978). This contrasts with F where the *tra* cistrons are contiguous (Figure 3.1); it may have been the result of a transposition or recombination event which occurred when a precursor of RP4 acquired kanamycin resistance.

In all, 19 *tra* mutants of RP4 were isolated, and this chapter deals with their further characterisation.

1. Transfer frequencies of *tra* mutants: Trimethoprim resistant transconjugants from the cross W3110T$^-$-Tn7(or J62-Tn7) x J53-2 were initially screened for their ability to transfer by plate matings with C600. Clones which showed reduced or no transfer by this method were retested in liquid matings as described in Chapter 1. The mating mixture was concentrated by centrifugation after one hour and samples containing at least 10^9 donor cells were plated onto a suitable selective medium. The results are shown in Table 3.1.

A reduced rate of plasmid transfer shown by some clones might have been caused by a high level of reversion from a Tra$^-$ to a Tra$^+$ phenotype. Transconjugants from such clones were purified and their transfer frequency measured again. In every case they showed the same reduced level of transfer as before and consequently their

Table 3.1. Properties of *tra* mutants of RP4 (Data from Barth, Grinter and Bradley, 1978)

Plasmid	Tn7 insertion site[1]	Phage sensitivity[2]			Piliation[3]: cells with		Transfer frequency (per donor per hour)	Complementation[4] group
		PRR1	Pf3	PR4	unlabelled pili (%)	PRR1-adsorbed pili (%)		
pRP46	30.9	S	S	S	-	-	10^{-4}	5
pRP92	30.2	S	S	S	1	65	10^{-4}	5
pRP76	29.5	S	S	S	-	-	10^{-6}	5
pRP90	29.3	S	S	S	4	49	10^{-6}	5
pRP89	29.1	S	S	S	-	-	10^{-5}	5
pRP67	28.2	S	S	S	5	38	$< 10^{-9}$	4
pRP69	28.2	R	R	S	0	1	$< 10^{-9}$	4
pRP68	28.1	R	R	R	-	-	$< 10^{-9}$	4
pRP74	28.0	R	R	R	0	0	$< 10^{-9}$	4
pRP66	27.6	R	S	S	0	31	$< 10^{-9}$	4
pRP81	27.6	R	R	S	-	-	$< 10^{-9}$	4
pRP75	27.5	R	R	S	-	-	$< 10^{-9}$	4
pRP77	27.5	R	R	S	4	12	$< 10^{-9}$	4
pRP79	27.4	R	R	R	-	-	$< 10^{-9}$	3
pRP78	27.3	R	R	R	-	-	$< 10^{-9}$	3
pRP26	27.2	S	S	S	-	-	10^{-5}	2

Table 3.1 Continued. Properties of *tra* mutants of RP4 (Data from Barth, Grinter and Bradley, 1978)

Plasmid	Tn7 insertion site[1]	Phage sensitivity[2]			Piliation[3]: cells with		Transfer frequency (per donor per hour)	Complementation[4] group
		PRR1	Pf3	PR4	unlabelled pili (%)	PRR1-adsorbed pili (%)		
pRP91	17.2	R	R	R	0	0	10^{-5}	1
pRP80	16.7	R	R	R	-	-	$< 10^{-9}$	1
pRP82	16.7	R	R	R	0	2	$< 10^{-9}$	1
Control: pRP3	20.0	S	S	S	14	53	10^{-3}	tra^+ control

The sensitivities to three different phages are shown in the table. PRR1 attaches to the sides of P-pili, Pf3 and PR4 to the tips (Bradley and Cohen, 1977). The pili behave as if they are retractile, but can be locked in an extended position by adsorption of PRR1, hence the increase frequently observed in levels of piliation detectable by electron microscopy after PRR1 adsorption. Resistance to a phage only means that cells are not lysed by that phage and does not imply the lack of an attachment site, e.g. clones carrying pRP66 and pRP77 are resistant to PRR1, but readily adsorb the phage. pRP nos. 69, 66, 81, 75 and 77 confer sensitivity to at least one of the pilus tip-specific phages, while remaining resistant to PRR1. This is consistent with their coding for pili which are normally retracted. The results also show that the tip-specific phages have different adsorption specifities.

1 This is the distance in Megadaltons from the single *Eco*R1 restriction site on RP4 (Jacob and Grinter, 1975) measured in a clockwise direction. The method of mapping the insertion sites is described in detail in Barth and Grinter, 1977.

2 These are determined by spot tests.

3 This is the percentage of piliated cells seen by electron microscopy both before and after treatment with the phage PRR1. - indicates not examined.

4 For explanation, see text and Table 3.2.

behaviour was not due to reversion.

The transfer of the *tra* mutants ranged from undetectable (less than 10^{-9} per donor per hour) to 10^{-4}. Any plasmid with a transfer frequency greater than 10^{-4} was considered *tra*$^{+}$.

2. Surface exclusion expressed by *tra* mutants: Tn7-generated *tra* mutants of RP4 in strain J53-2 isolated as described above were tested for their ability to exclude the entry of two other IncP plasmids, R1033 and R702.

Broth matings were carried out for one hour as described in Chapter 1. The mating mix was plated onto minimal medium containing the nutritional requirements of J53 plus either chloramphenicol (selecting for R1033 transfer) or sulphonamide (selecting for R702 transfer). Surface exclusion negative (Sex^{-}) recipient controls were R^{-} J53 and (RP4δ1)J53.[1] Sex^{+} controls were (RP4)J53 and (pRP3)J53.[2]

Initial experiments with R1033 suggested that all the *tra* mutants were Sex^{+} (i.e. indistinguishable from RP4 and pRP3). However, R1033 was only slightly susceptible to exclusion by RP4, there being only a tenfold difference between the number of colonies with the Sex^{+} and Sex^{-} recipient controls. The experiments were repeated using R702 as donor. This was excluded by RP4 as much as fivefold more than R1033. (P. T. Barth, unpublished data, has since shown that R751, another IncP plasmid, is excluded as much as 100 times more efficiently by RP4 than R1033). The results with R702 confirmed the earlier conclusion that none of the *tra* plasmids were Sex^{-}.

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1 RP4δ1 is a spontaneous deletion mutant of RP4 which has simultaneously lost surface exclusion and kanamycin resistance (Hedges *et al.*, 1976).

2 pRP3 is a Tn7 derivative of RP4 which, apart from carrying the additional antibiotic resistances, confers a phenotype indistinguishable from that of the parent plasmid (Barth and Grinter, 1977).

[The recipient strain had a marked effect on the expression of surface exclusion. Comparisons showed that (RP4) *E. coli* C was 100 times more efficient as a recipient of R1033 (in strain W3110T$^-$) than (RP4)J53. This result was not due to instability of RP4 in *E. coli* C.]

3. Complementation between the *tra* mutants. If a *tra* mutant, unable to produce a particular, diffusible protein necessary for transfer, is present in the same cell as another *tra* mutant which makes this protein, then transmissibility will be to some extent restored. This is the basis of a complementation test. It allows *tra* plasmids to be put into groups which are defective in the same function (and consequently, non-complementary).

There are three obstacles to constructing strains heterozygous for RP4 *tra* mutants:

i) Many mutants have no detectable transfer.

ii) Plasmids with residual transfer capacity will be excluded by the resident *tra* plasmid.

iii) Incompatibility between the mutants will make the heterozygote unstable.

These problems were overcome at least in part by a modification of the P1 transductional complementation system described by Willetts and Achtman (1972). Bacteriophage P1 was used to transduce the plasmids (simultaneously overcoming their lack of transmissibility and surface exclusion by the plasmid in the recipient) and the heterozygotes thus formed were kept in conditions where cell division and hence segregation were unlikely.

P1 lysates were prepared from strains carrying the *tra* mutants of RP4. The lysates were checked for sterility and titrated for plaque-forming units (Chapter 1). Cultures of the recipients containing *tra* plasmids were grown with aeration in broth with 10 mM

$CaCl_2$ to a titre of 5 x 10^8 cells per ml and concentrated tenfold by centrifugation and resuspension in the same medium. 0.1 ml was mixed with 0.1 ml of P1 lysate (diluted in P1 buffer to give a multiplicity of infection of 1) and adsorption allowed for 15 minutes at 37°C. 0.1 ml of sodium citrate in broth (3% w/v) was added to chelate the Ca^{++} ions and terminate adsorption, followed by 0.1 ml of strain C600, grown in broth with aeration to 5 x 10^8 cells per ml and concentrated tenfold by centrifugation and resuspension in broth containing sodium citrate (1% w/v).

The mixture was left for 2 hours at 37°C for expression of the transduced plasmids to take place. During this time, complementary pairs of plasmids in the transductional recipient could bring about transfer by conjugation to C600. The mixture was then plated on minimal medium containing sodium citrate (1% w/v), the nutritional requirements of C600 and kanamycin (25 µg/ml to select transconjugants).

Several control experiments were necessary:

1. To check that sodium citrate did not inhibit conjugation, two "complementation" experiments were carried out, substituting phage buffer for P1 and having a *tra*$^+$ RP4-Tn7 plasmid in the "transductional recipient". Sodium citrate was omitted from one of the crosses. No difference was found in the number of transconjugants obtained in the two crosses (although sodium citrate in the plates did seem to reduce the colony size of the transconjugants).

2. To test the efficiency of sodium citrate as an inhibitor of P1 adsorption, transductions of J53 were carried out in the presence of 1% w/v citrate using a P1 lysate grown on (RP4)J53. Transduction was completely undetectable (i.e. reduced by a factor greater than 10^4). However, if a complementation experiment was carried out using a P1 lysate grown on a strain containing the same *tra* plasmid as that

in the transuctional recipient, some colonies were seen on the plates selective for the C600 conjugative recipient. These colonies could not be due to complementation and so it was assumed that they resulted from the transductional recipient acting as a secondary source of transducing particles. This was apparently confirmed because the number of colonies observed was unchanged when a P1 donor grown on an R^- strain was used, and no colonies were observed when P1 was omitted (i.e. there was no residual level of conjugative transfer coded by the *tra* plasmid under test). In consequence, the number of colonies produced by P1 grown on an R^- donor was measured with each *tra* plasmid in the transductional recipient, and subtracted from the number of colonies produced in a complementation.

Subsequently it was shown that the colonies produced when the transductional donor was R^- were spurious. Attempts to purify them on MacConkey medium containing kanamycin (10 µg/ml) gave no growth whatsoever, whereas most colonies produced from complementary crosses could be purified on this medium. Possibly the presence of high concentrations of P1 or the presence of citrate somehow protects some cells against the action of kanamycin.

Whatever the mechanism of formation of these spurious colonies, they were allowed for by the subtraction of the number of colonies produced when an R^- strain was the transductional donor. Generally the numbers of such colonies were less than 10% of those from a complementing cross. When pRP80 was the plasmid in the transductional recipient, however, the percentage of these colonies was consistently higher (up to as much as 50% of the total from a complementing cross). The reason for this is not understood.

3. When the transductional donor contained a plasmid with a residual level of transmissibility, the number of transconjugants due to this

transfer had to be estimated and subtracted from the total to give the number of conjugative transfers resulting from complementation. This was measured by using R^- J53 as a transductional recipient and counting the number of transfers to C600 by conjugation. This was then subtracted from the colony numbers arising from a complementation test. [It was not possible to use the transfer-defective plasmids as transductional recipients since the residual level of conjugative transfer was too high to allow detection of an increase due to complementation].

4. All experiments were carried out in Rec^+ strains and so some transfers could be due to recombination (which could be intragenic) rather than complementation. About 100 colonies from complementing crosses (involving pRP78 or 79 and pRP80 or RP4δ1, Table 3.2) were purified and tested for their subsequent transferability. Only one was found which was Tra^+ (and Tp^S) and so recombination was only rarely responsible for transfer even when the mutations were widely separated as here. Consequently, the contribution of intragenic recombination must be very low indeed.

The results of the complementation experiments are shown in Table 3.2. This shows that the *tra* mutants fall into five groups. Members of a group do not complement each other, but complement members of other groups. pRP82, 80 and 91 form group 1; pRP26 is the only member of group 2; pRP78 and 79 make up group 3; pRP77, 75, 81, 66, 74, 68, 69 and 67 form group 4; pRP89, 90, 76, 92 and 46 constitute group 5. Not all possible crosses were attempted. Some were not possible because of the residual transmissibility of the plasmids involved (see above) and in other cases, only representatives of one group were tested against representatives of another. Also the mapping positions of the mutants were taken into account. For example, pRP78 (map position 44.7, Table 3.1) complements pRP77(43.7). It is therefore

Table 3.2. Transductional complementation data

pRP plasmid in P1 donor	pRP plasmid in P1 recipient (conjugative donor)												
	67	69	68	74	66	81	75	77	79	78	80	82	RP4δ1
46	c 200									c 900			
92	c 200									c 500			
76	c 40									c 80			
90	c 1000									c 1900			
89	c 600									c 1200			
67	0	0	0	0	0	0	0	0	88				85
69	0	0		0				0					
68	0		0	0				0					57
74	0	0	0	0				0		234			
66	0				0			0	779	702		681	468
81	0					0		0					
75	0						0	0					
77	0	0	0	0	0	0	0	0		98			
79	51								0	0			

Table 3.2 Continued. Transductional complementation data

pRP plasmid in P1 donor	pRP plasmid in P1 recipient (conjugative donor)												
	67	69	68	74	66	81	75	77	79	78	80	82	RP4δ1
78				105				152	0	0		44	22
26	c 900							c 900		c 900		c 900	50
91										c 500		0	
80											0	1	
82										175	0	1	1
RP4δ1	5									10		0	

Crosses were performed as described in the text. The plasmids are listed in mapping order (Table 3.1). The numbers in the table are colonies per 10^9 P1 p.f.u. after subtraction of the appropriate control figures. Numbers prefixed c. were calculated from large colony numbers which could only be approximated. The low numbers for RP4δ1 complementary crosses where it was in the P1 donor are probably a reflection of its low transduction frequency (2×10^{-7} as compared with 5×10^{-6} for the other plasmids).

to be expected that pRP78 will complement all more distal mutations on the same side as pRP77, and results with other crosses bear this out.

The possible relationship between complementation groups and *tra* genes is discussed below.

4. Testing the ability of piliated *tra* mutants to mobilise a non-conjugative plasmid. F has an origin of transfer (see introduction) and recent work has shown that a similar structure exists on plasmids which do not encode their own transfer system. Possession of such a site allows these plasmids to be mobilised by other, conjugative plasmids, and mutants can be isolated (*mob*$^-$) which have lost this ability (Warren and Sherratt, 1977). Some of the *tra* mutants of RP4 produced pili almost as frequently as the *tra*$^+$ control (see Barth *et al.*, 1978) and yet showed no detectable transfer. If Tn7 insertion had disrupted only the origin of transfer in these plasmids, they should retain the ability to mobilise a non-conjugative plasmid.

The plasmids chosen to test this all came from complementation group 3, which has the largest number of members and is also the most heterogeneous with regard to phage sensitivity and degree of piliation. pRP67 confers sensitivity to all three pilus specific phages tested (Table 3.1) and pili are detectable by electron microscopy at a frequency greater than one third of that produced by a *tra*$^+$ control (even in the absence of adsorbed pilus-specific phage which boosts the number of detectable pili, see Barth *et al.*, 1978). pRP66 confers sensitivity only to both phages which attach to the pilus tip (Table 3.1), as if strains carrying this plasmid produced retracted pili. Electron microscopy revealed no pili on untreated cells. Labelling with PRR1, which however, (a pilus-specific phage attaches to the side of P pili, Bradley and Cohen, 1977) gives a detected level of piliation greater than one half of that seen in a similarly treated *tra*$^+$ control. pRP77

is sensitive only to one of the tip attaching phages (Table 3.1) and labelling with PRR1 before electron microscopy gives detectable piliation at less than 25% of the control. pRP74 is fully resistant to pilus-specific phages and no pili can be detected by electron microscopy even after PRR1 treatment.

All four plasmids failed to transfer at a detectable frequency (see section 1). Cultures of each in J53-2 were transformed (Chapter 1) using purified DNA of R300B, a 5.7 Md. non-conjugative plasmid coding for streptomycin and sulphonamide resistance (Barth and Grinter, 1974). Also transformed were (pRP3)J53-2[1] and R^- J53-2 to act as positive and negative controls.

After purification, a representative of each class was crossed with J62 in broth and plated on minimal medium containing the nutritional requirements of J62 and either streptomycin (15 µg/ml) or kanamycin (25 µg/ml) to select for transfer of either R300B or the mobilising plasmid.

There was no detectable transfer except from (R300B)(pRP3)J53-2 which produced roughly equal numbers of Sm^R and Km^R colonies (representing a transfer frequency of 5-10 x 10^{-4} per donor per hour). Consequently, none of the *tra* plasmids tested behaved as if they had lost only the origin of transfer. Further tests are planned to see if the *tra* plasmids can be mobilised by the introduction of a distinguishable IncP plasmid by transduction. This should allow detection of those mutants which have lost the origin of transfer even though they may have simultaneously lost other functions. This work has not yet been undertaken.

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1 A *tra*$^+$ Tn7 derivative of RP4.

Discussion

The results presented here are from a preliminary investigation and many factors make their interpretation difficult. As has been reported (Barth and Grinter, 1977), the insertion of Tn7 into RP4 often caused deletions. It has been shown by heteroduplex analysis (G. Engler, M. Van Montagu and J. Schell, personal communication) that these deletions are of material from the recipient plasmid and occur at the site of Tn7 insertion. Also, Tn7 insertion may have polar effects on distal genes within the operon in which it is inserted, like those observed with Tn1 (Heffron *et al.*, 1975) and Tn10 (Kleckner *et al.*, 1975). Further, the phage sensitivity conferred by a particular *tra* mutant of RP4 does not necessarily correlate with the presence or absence of phage attachment sites. Resistance to a particular, pilus-specific phage means only that the strain is not lysed by that phage. For example, pRP66 and 77 (Table 3.1) are resistant to phage PRR1, but electron microscopy of cultures carrying these plasmids showed that PRR1 could attach to their pili, and the number of PRR1 labelled pili seen in cultures containing pRP66 was similar to that in the tra^+ control. Finally, transmissibility is not necessarily correlated with detectable piliation. pRP67 confers sensitivity to all three pilus-specific phages and gives nearly normal numbers of pili seen by electron microscopy, but its transfer is undetectable. In contrast, pRP91 does not confer visible phage sensitivity or detectable pili, but transfers at a frequency 1% of that of the tra^+ control.

All the plasmids tested which determined production of detectable pili showed an enhanced level of piliation after treatment with PRR1. This suggests that pili are normally only rarely extruded and that PRR1 adsorption locks the pili in an extended position (Bradley and Cohen, 1977). Where plasmid-carrying strains are sensitive only to

Pf3 and PR4 (which are thought to attach to the tips of P-1 pili, Bradley and Cohen, 1977), this is consistent with their pili being more or less permanently retracted. pRP66 may be an example of this. Cells carrying it have no visible pili without PRR1 labelling (Table 3.1). [pRP69, 81 and 75 may belong to this category, although their pili were not examined microscopically. Cells bearing these plasmids are $PRR1^R$, $Pf3^R$ and $PR4^S$ (Table 3.1). This shows the two tip-specific phages have different attachment or infection specificities. pRP77 gives a similar phage resistance pattern, but pili are visible on cells carrying it even without PRR1 labelling].

The *tra* plasmids can be divided into 8 groups on the basis of conferred phage sensitivity and map position. Group 1 consists of pRP46, 92, 76, 90, 89 and 67. These could be a series of polarity mutants, all affecting the same gene. However, the expected polarity gradient is not seen. (The question of polar mutations is discussed more fully in Chapter 4.) Also, the complementation data (Table 3.2) show that pRP67 belongs to a different group from the others. However, none of the plasmids in this phenotypic group are markedly affected in their ability to produce pili, suggesting they may be defective in forming stable mating aggregates, in triggering of plasmid DNA replication or in DNA transfer (assuming that conjugation involves the same stages as seen with F).

Phenotypic group 2 (pRP69) confers sensitivity only to the tip-specific phage PR4, and has only 1% of cells detectably piliated even after PRR1 treatment. This suggests the pili are either not being efficiently assembled or are almost always retracted and that the *tra* mutation has resulted in inability to form cell-to-cell contacts.

Group 3 (pRP68 and 74) cultures are fully phage resistant and, where tested, no pili are visible even after labelling with PRR1.

Like group 2, the mutation has probably abolished mating aggregation.

Group 4 (pRP66) cultures, as suggested above, may possess retracted pili.

Group 5 (pRP81, 75 and 77) cultures are sensitive only to PR4. Where tested, however, (pRP77) the pili are not retracted, but are present in lower numbers than in the control. The plasmids in this group may code for defective pili or be altered in some regulatory function which has caused repression of the *tra* genes. [Several P plasmids have been shown to have their transfer repressed by the presence of an IncIα plasmid in the same cell (Datta *et al.*, 1971). This may not be analogous to the *finOP* system of F, since P pilus production is essentially unaffected by the presence of the IncIα plasmid (N. Datta, personal communication).]

Group 6 (pRP79 and 78) cultures are resistant to all three phages and may be unable to produce pili.

Group 7 (pRP26) cultures are sensitive to all three phages. The reduced level of transfer, as with most members of group 1, may be due to defective aggregation, triggering or transfer of DNA.

Group 8 (pRP91, 80 and 82) cultures are resistant to the pilus-specific phages. Only pRP82 codes for detectable pili (1 piliated cell out of 100 after PRR1 treatment). A mutation in a control gene could explain this result, although it is puzzling that pRP91 transfers so well. Possibly two separate mutations are covered by this group, one (pRP80 and 82) in a control gene and one (pRP91) which alters pilus morphology sufficiently to prevent phage adsorption and detection by electron microscopy, but still leaves a residual ability to establish cell-to-cell contact and to transfer DNA. This is compatible with the complementation data (which puts pRP91 in the same complementation group as pRP80 and 82) only if the control gene regulates

the expression of the whole *tra* region containing this group. The mutations in pRP80 and 82 must either lead to the production of a tight-binding repressor of *tra* functions, or must result from the loss of a binding site for a positive control protein. The data for pRP1 (Table 3.1) show that the *tra* genes of RP4 are not normally repressed, unlike F. Moreover, any positive control protein coded by complementation group 1 can only regulate the genes within that group, otherwise the loss of its binding site would mean the mutant would be unable to complement other *tra* mutations. This would also contrast with F where nearly all the *tra* genes are under common control (see Introduction).

In the absence of further data, the interpretation of the phage sensitivities, degree of piliation, and transfer frequencies remains highly speculative.

The result that none of the *tra* mutants had lost the ability to exclude a superinfecting IncP plasmid is explained by a consideration of RP4δ1. This is a spontaneous deletion mutant of RP4 (Hedges *et al.*, 1976) which has simultaneously lost kanamycin resistance and surface exclusion. Complementation data show (Table 3.2) that this plasmid retains all the *tra* functions except those encoded in the region between 16.7 and 17.2 Md on the RP4 map (Table 3.1 and Barth and Grinter, 1978). Therefore the 9 Md. deletion suffered by RP4δ1 must cover approximately the 15-24 Md region of RP4. Stanisich and Bennett (1976) showed that the Tra and Sex determinants of RP1 (a plasmid indistinguishable from RP4) were closely linked (about 1 Md. apart). Therefore the surface exclusion genes must lie somewhere in between 15 and 16.7 Md. on the RP4 map. [Subsequent work by P. T. Barth (unpublished data) has led to the isolation of Tn7 derivatives of RP4 which are Sex$^-$ and which map in this region. They are

also wholly or partially Tra$^-$].

The finding that there is a hierarchy in the expression of surface exclusion (RP4 excludes R751 more than R702, and R702 more than R1033) suggests that, like F, surface exclusion is controlled by more than one gene and some functions are plasmid specific.

Turning to the complementation data, although precise interpretation is again difficult, the existence of 5 complementation groups indicates that at least five functions have been separately eliminated by Tn7 insertion and so at least 5 genes are involved in RP4 transfer.

The complementation data fit the *tra* mutants into groups consistent with the mapping data (see Table 3.1). Groups 1, 2, 3 and 4 each map within a length of DNA consistent with their being a single gene. Group 4, however, contains 5 phenotypic classes (see above), some having normal pili, some retracted pili, and some having no detectable pili. It is just possible that all the mutants in group 4 result from insertions in a single gene which is responsible for assembly of a functional pilus, but without a more detailed knowledge of how Tn7 insertion disrupts the surrounding genetic material and affects its subsequent expression, this is only guesswork.

Finally, it is worth considering host ranges. RP4 and most other IncP plasmids have a uniquely wide host range, whereas that of F and F-like plasmids is more restricted (Jacob *et al.*, 1977a). A wide host range may mean that a plasmid lacks specialised replication and segregation controls which would make it particularly suited to one particular group of hosts (and simultaneously unsuited to others). A restricted host range, on the other hand, may mean that a plasmid is highly adapted to transfer between and replication in one group of hosts. It is therefore possible that RP4 has a much simpler transfer system than F. Certain hosts, however, by their unusual internal

environment, may impose a powerful selective pressure upon any wide host range plasmids infecting them, forcing the plasmids to become more specialised.

However, the opposite may be true. RP4 and most other P plasmids may have the ability to transfer between and replicate in many hosts as a result of carrying many genes for transfer and replication, only some of which are essential in one particular genus. (This might explain why there are regions in RP4 *tra* genes where no insertions were detected. These regions may not affect transfer between *E. coli*, but might be important in other genera.)

An indication of the complexity of a plasmid's transfer system is given by the size of its *tra* region. In F, the *tra* genes occupy about a third of the molecule (c 20 Md. or roughly 30 genes, see Figure 3.1), whereas in RP4 the two *tra* regions are probably contained within the space of 10 Md. (c 15 genes) assuming there are no other *tra* regions elsewhere (Barth *et al.*, 1978 and unpublished observations). This suggests that the RP4 *tra* system is probably simpler than F. Consistent with this is the lack of pilus suppression coded by RP4, a feature shared with IncW plasmids which are the smallest (and hence probably the simplest) conjugative plasmids.

In conclusion, the work presented in this chapter is only a beginning. *tra* mutants of RP4 affecting pilus synthesis, possible control genes and other functions have been isolated, mapped and characterised, and at least five genes have been shown to be involved in transfer.

CHAPTER 4

PLASMIDS AS VECTORS FOR TRANSPOSONS

Abstract

Certain DNA segments (transposons and insertion sequences) have been identified through their ability to transpose between replicons, the DNAs of which show little homology. One such sequence, transposon 7 (Tn7), found originally as part of the IncIα plasmid R483, contains genes coding for resistance to trimethoprim and streptomycin. The experiments described in this chapter characterise several aspects of Tn7 transposition:

1. Specificity of insertion into the *E. coli* chromosome. A highly preferred or unique site was detected and a rough estimate made of the size of Tn7.
2. The frequency of transposition from the *E. coli* chromosome to RP4 was measured.
3. Several techniques were used in an attempt to insert Tn7 into a small, non-conjugative plasmid.
4. Tn7 derivatives of RP4 which no longer conferred resistance to either kanamycin or tetracycline were tested for their ability to revert to a drug-resistant phenotype.
5. *In vitro* manipulations generated several derivatives of Tn7 which had undergone rearrangement and/or deletion. These were tested for their ability to transpose.
6. Transposition was found to be reduced in certain strains, suggesting that host functions are involved in the process.

The mechanism of transposition and its role in nature are considered.

Introduction

1. Discovery and incidence of transposable DNA sequences

Transposable DNA sequences were first recognised in maize (McClintock, 1952) where control elements affect the functioning of various loci and behave as discrete genetic determinants. (For a review, see Fincham and Sastry, 1974.) Many similar examples have been detected in other eukaryotic systems (Bukhari *et al.*, 1977, Section V) although their precise nature remains indeterminate because of their complexity and other inherent technical difficulties.

The first clear evidence for transposable DNA sequences in bacteria came from the analysis of an unusual class of Gal$^-$ mutants first described by Lederberg (1960). In these strains, the levels of expression of three different enzymes involved in galactose utilisation had been simultaneously reduced to less than 1% of that in the wild type. Deletion mapping (Shapiro and Adhya, 1969) of mutations causing a galactokinase negative phenotype showed that only some of the mutations had occurred in the *galK* gene. Other mutations mapped in the *galT* and *galE* genes, or in the control region of the *gal* operon. The Gal K$^-$ phenotype resulted from polarity effects of these mutations. This polarity was much more severe than that caused by amber mutations in *galE* or *galT* (Jordan and Saedler, 1967; Adhya and Shapiro, 1969), suggesting that a different mechanism was involved.

The mutants were found to revert spontaneously to Gal$^+$ at a frequency of 10^{-6} to 10^{-8}, and the reversion frequency was not enhanced by mutagens. This showed that the mutations were not deletions or point mutations and that other possible mechanisms had to be considered.

Measurement of the density of λ*gal* transducing phages (Jordan *et al.*, 1968; Shapiro, 1969) showed the strongly polar *gal* mutants to have extra DNA in the *gal* operon, while Gal$^+$ revertants had normal DNA

contents. (This meant that the mutants were not the result of a genetic inversion). To determine the origin of the extra DNA, Michaelis *et al.* (1969) repeatedly hybridised RNA transcribed *in vitro* from mutant λ*gal* phages to the DNA of λ*gal* phages not carrying the mutation. There was always a fraction of RNA which specifically hybridised to the DNA of the mutant phage, showing the extra DNA in the mutant did not originate from within the *gal* operon (i.e. it was the result of an insertion of material from outside the *gal* region, rather than an internal duplication).

Heteroduplex analysis allowed the visualisation of the inserted sequences and showed that some were homologous with each other (Fiandt *et al.*, 1972; Hirsch *et al.*, 1972a and b; Malamy *et al.*, 1972). The five best characterised insertion sequences have been designated IS1-IS5, but many other sequences with similar properties have been identified and await classification (see Szybalski, 1977).

Insertion sequences have been found on a variety of replicons. Saedler and Heiss (1973) detected approximately 8 copies of IS1 and 5 of IS2 in the *E. coli* K12 chromosome. IS1 (but not IS2) is also found in the chromosome of *Salmonella typhimurium* LT2 (Starlinger and Saedler, 1976). Many plasmids carry insertion sequences: on F they are involved in Hfr and F' formation (Hu *et al.*, 1975b), and they are found on other plasmids often at the junctions between RTF and r - determinant regions (Hu *et al.*, 1975a; Cohen, 1977; Ohtsubo and Ohtsubo, 1977). Chow (1977) has studied the organisation of the *E. coli* chromosome and detected structures like insertion sequences arranged in a regular manner. As much as 18% of the chromosome may be divided into regions of two characteristic lengths bounded by these IS-like segments. The significance of the widespread occurrence of insertion sequences and their possible role in the evolution of

plasmids and chromsomes is discussed below.]

Insertion sequences are generally shorter than 2 kilobases (enough DNA to code for 2-3 proteins) and so far no IS-coded products have been detected. Their ability to transpose is detected by their mutagenic effect in the cistrons into which they insert. A new class of transposable sequences which carry recognisable genes was first described by Hedges and Jacob (1974). They discovered a DNA segment of approximately 3 Md. coding for ampicillin resistance, which was able to transpose from its parent replicon, the plasmid RP4, to the *E. coli* chromosome and to other plasmids. This was termed a "transposon" (transposon A or TnA, now called Tn1) and the authors noted the similarities with insertion sequences. Many other transposons have since been identified and these code for a variety of functions (Table 4.1).

2. Properties of transposable DNA sequences

a) Structure. Examination by electron microscopy of single-stranded DNA molecules has revealed that transposons are bounded by short (< 2 kilobases) complementary DNA sequences which may be in either orientation with respect to each other (Ptashne and Cohen, 1975). These repeated sequences are of varying length (140-1500 base-pairs, see Kleckner, 1977). In two cases they have been shown to be homologous with IS1: in Tn9 as a direct repetition (Machattie and Jackowski, 1977) and in Tn1681 as inverted repeats (So *et al.*, 1979). [The inverted repeats at the ends of Tn10 were thought to be IS3, but this is no longer believed to be true (Kleckner and Ross, 1978).] DNA sequencing of IS1 and IS2 has also revealed the existence of internal inverted repeats (Ohtsubo and Ohtsubo, 1978; Ghosal and Saedler, 1978) and such structures must be important in determining transposability.

Table 4.1. Transposons and their properties

Transposon	Phenotypic marker	Molecular weight (Md.)	Origin	Reference
Tn1	Ap	3	RP4	Hedges and Jacob (1974)
Tn2	Ap	3	RSF1030	Heffron *et al.* (1975)
Tn3	Ap	3	R1	Kopecko and Cohen (1975)
Tn4	ApSmSu	13.6	R1	Kopecko and Cohen (1975)
Tn5	Km	~ 3.5	JR67	Berg *et al.* (1975)
Tn6	Km	~ 2.6	JR72	Berg *et al.* (1975)
Tn7	TpSm	9	R483	Barth *et al.* (1975)
Tn9	Cm	~ 2	pSM14	Gottesman and Rosner (1975)
Tn10	Tc	~ 6	R100	Foster *et al.* (1975), Kleckner *et al.* (1975)
Tn501	Hg^{++R}	5.2	pVS1	Stanisich *et al.* (1977)
Tn951	Lac^{+} (*lac i, z* and *y*)	~11	pGC1	Cornelis *et al.* (1978)
Tn1681	S.T. + Tc^{1}	~ 3	ESF0041	So *et al.* (1979)
Tol	Toluene-degradation	38	Tol. plasmid	Jacoby *et al.* (1978)

1 Tn1681 is a laboratory construct. Non-transposable tetracycline-resistance genes were inserted *in vitro* into the genes for heat-stable toxin (S.T) which had a structure typical of a transposon when viewed under the electron microscope. Transposition of the composite transposon could now be monitored using Tc^{R}.

b) Transposition is distinct from general (homologous) recombination. Transposition is a non-reciprocal event which requires neither a functional *recA* gene nor extensive DNA homology between donor and recipient replicons (Starlinger and Saedler, 1976; Kleckner, 1977).

c) Mutagenesis. Transposition, by interrupting a pre-existing gene sequence or control region, produces a mutation.

d) Effects on genes distal to the site of insertion. Insertion sequences were originally identified because they could co-ordinately reduce the expression of several genes in the operon into which they were inserted (see above). IS1 gives rise to such polarity effects in either orientation (Fiandt *et al.*, 1972), while IS2 does so in one orientation only (orientation I). [IS2 in orientation II acts as a powerful promoter, leading to constitutive levels of expression of distal genes in the operon in which it is inserted (Saedler *et al.*, 1974).] Starlinger *et al.*, 1973, showed that messenger RNA synthesis was blocked distal to polar insertion mutations, suggesting that transcriptional termination signals were present on the inserted DNA.

A model for transcriptional termination (Adhya *et al.*, 1976) proposes that after ribosomes dissociate from nascent messenger RNA beyond a polypeptide terminator codon (UAA, UAG and UGA), transcription continues to the next site on the DNA which is recognised by the transcription terminator protein, rho. If ribosomes reinitiate protein synthesis beyond the nonsense codon and before the rho site, rho is overridden and transcription continues. Polarity results when a nonsense codon triggers ribosome dissociation in a region devoid of translation restart signals. In this case, cistrons beyond the next rho site are not transcribed.

The effects on polarity of rho and t-RNA supressor mutations suggest that IS2 (in orientation I) has nonsense codons and a rho site,

whereas IS1 (in both orientations) carries nonsense codons and no translation reinitiation codons (Besemer, 1977; Das *et al.*, 1977).

Polarity effects have been observed with other insertion sequences (Szybalski, 1977) and some transposons [Rubens *et al.*, 1976; Heffron *et al.*, 1977a (Tn1, Tn2 and Tn3); Berg, 1977 (Tn5); Kleckner *et al.*, 1975; Foster, 1977 (Tn10)]. Although the polar effects of transposons have not been as fully investigated as those due to insertion sequences, transcriptional termination may be involved in at least one case (Tn10, Kleckner *et al.*, 1978).

e) Promotion of genetic aberrations. Many of the well-characterised transposable sequences promote the formation of deletions affecting adjacent chromosomal material. Among the insertion sequences, the best documented case is that of IS1. Deletion formation by IS1 (which does not require *recA*) is strongly temperature dependent and can be reduced by a factor of as much as 10^4 at 42°C as compared with 32°C. Several classes of deletions are formed. One end point of most deletions is at the terminus of IS1, and the other point occurs at different sites on the adjacent chromosome, but these sites are not randomly distributed. Deletion is thus of a continuous segment of DNA extending outwards from one end of IS1 into the surrounding chromosome, leaving the IS1 element itself intact at the end of the deleted section (Reif and Saedler, 1975 and 1977). Deletions promoted by IS2 and IS3 appear to be similar in form, although very few examples have been examined thus far (Hu *et al.*, 1975a; Ohtsubo and Ohtsubo, 1977; Davidson *et al.*, 1974). Deletion formation by IS2 is not temperature dependent and so may have a different mechanism from that of IS1 (Saedler, 1977).

Most transposons have also been shown to promote unusual recombinational events in the absence of a functional *recA* gene.

Tn1, Tn3 and Tn9 have been shown by genetic and physical experiments to produce deletions extending from one end of the transposon into the adjacent chromosome, leaving the transposon intact. The frequency of deletion formation seems to depend on the recipient replicon (Heffron *et al.*, 1977a). In at least some cases, these deletions occur after insertion of the transposon and are therefore due to a separate event. Tn9 can also generate tandem duplications of itself (Nisen *et al.*, 1977; Weinstock and Botstein, 1978; Rosner and Gottesman, 1977; Machattie and Jackowski, 1977). With Tn7, on the other hand, deletion frequently occurs on insertion of the transposon, causing loss of up to 3 Md. of DNA from the recipient replicon (Barth and Grinter, 1977; Barth *et al.*, 1978; Results, this chapter). Transposition of Tn5 and Tn10 does not normally lead to deletion of material from the recipient molecule (Botstein and Kleckner, 1977) but a variety of Tn10-mediated post-insertional deletions, rearrangements, inversions and duplications have been detected. Sometimes these events involve only adjacent chromosomal material and sometimes they take place entirely within the transposon itself. On other occasions, DNA both inside and outside Tn10 is involved (Noel and Ames, 1978; Ross *et al.*, 1979).

The mechanism of these recombination events and their relationships to one another are not understood, although it is clear that they occur by a pathway independent of that responsible for general homologous recombination. It is also clear that most of the special recombination events promoted by transposons occur at particular sites on the transposons themselves, usually their ends (Heffron *et al.*, 1977b; Ross *et al.*, 1978; Nisen *et al.*, 1977; Kleckner *et al.*, 1978).

Mutations mapping close to *recA* have been isolated which affect deletion formation by IS1 (Nevers *et al.*, 1977). IS1 could still excise normally (see below) in these strains, showing that excision

and deletion-generation are separate processes. Further characterisation of these mutants and the isolation of others affecting deletion formation by other transposable sequences should lead to a better understanding of deletion generation.

f) Specificity of insertion. Where studied, most transposable DNA sequences exhibit at least some specificity of insertion. The exceptions to this are Tn5 which seems to have no preferred sites of insertion at least within the *lacZ* gene (Berg, 1977), and the temperate bacteriophage Mu which seems to insert randomly into plasmids and the *E. coli* chromosome (Denarié *et al.*, 1977; Bukhari *et al.*, 1977).

Insertion sequences exhibit hot spots for insertion (Pfeifer *et al.*, 1977; Kuhn *et al.*, 1979). Five examples of IS1 insertion sites have been sequenced (Calos *et al.*, 1978; Grindley, 1978) and it is clear that the insertions are not into any specific sequence, nor is there any homology between the target site and the ends of IS1. A relationship between the target sites is not, however, disproved (Grindley, 1978); alternatively the sites of insertion may be at some distance from a recognition sequence.

Tn1, Tn2 and Tn3 have been shown to insert non-randomly into RSF1010 (Rubens *et al.*, 1976; Heffron *et al.*, 1977a) as has Tn1 into ColE1 (Dougan and Sherratt, 1977; Inselburg, 1977). Tn3 also has hot spots for insertion into R6-5, one of which is IS1 (Kretschmer, 1977). Tn7 is unusual in that it has a unique or highly preferred site of insertion in the *E. coli* chromosome, and apparently it has no insertion sites on some plasmids (Barth *et al.*, 1976, and Results, this chapter). In spite of this, it inserts into RP4 at a large number of sites (at least 40) with only a low level of specificity (Barth and Grinter, 1977; Barth *et al.*, 1978).

The insertion of Tn10 into the *lac* and *his* operons has been

extensively studied, and hot spots detected (Botstein and Kleckner, 1977; Foster, 1977; Noel and Ames, 1978). Insertions are evenly distributed between the clusters and there is no bias towards either orientation of insertion within a cluster (Kleckner *et al.*, 1979). The lack of a preferred orientation is shared by Tn1, Tn2 and Tn3 (Heffron *et al.*, 1977a) in sharp contrast to Tn7 which seems to insert into RP4 exclusively in one orientation (Barth and Grinter, 1977). [This specificity may relate to the direction of transcription. F. Cannon (personal communication) has mapped Tn7 insertions in the *nif* genes from *Klebseilla* and finds the orientation is always the same with respect to the direction of transcription.]

Apart from the actual DNA sequence of the recipient replicon, other factors may influence the site of insertion. The target site must be accessible to enzymes involved in transposition, and essential functions of the recipient replicon must not be inactivated as a consequence of the insertion. Grinsted *et al.* (1978) have shown that the presence of one transposon in the recipient molecule influences the specificity of insertion of another, possibly by introducing conformational changes. In addition, the presence of one transposon in the recipient molecule severely inhibits its ability to acquire another copy of the same transposon. This kind of "superinfection inhibition" may exist between all related transposons and may serve to limit their mutagenic action. It is exclusively a *cis*-effect and its mechanism is unknown (Robinson *et al.*, 1977).

g) Excision of transposed DNA. If an insertional mutation can revert by excision of the foreign DNA, this indicates that the recipient DNA sequences were not damaged as a consequence of insertion. Clean excision of IS1 has been shown to restore activity to the gene sequence which was interrupted (Reif and Saedler, 1975) and Tn5 exhibits similar

properties (Berg, 1977). Insertional mutations caused by Tn1 do not revert, however, but polar mutants do (Hernalsteens *et al.*, 1977). This means either that insertion of Tn1 gives rise to deletion of DNA from the recipient or that excision is not a precise cutting out of the transposon sequence. Tn1 insertion is known to generate detectable deletions on occasions (Heffron *et al.*, 1977a), so this could be the explanation.

Tn7-generated mutations are rarely revertible which is in agreement with the observation of deletions which are often formed on insertion of the transposon at the site of the insertion (Barth *et al.*, 1978; G. Engler, M. Van Montagu and J. Schell, personal communication, Results, this chapter).

Tn9 is unusual in that the DNA sequences at its ends are directly repeated. Possibly as a consequence of this, excision of Tn9 leaves behind one copy of the repeated sequence (IS1) and does not restore activity to the mutated gene (Rosner and Gottesman, 1977; Machattie and Jackowski, 1977).

Insertion of Tn10 does not seem to damage the recipient DNA since the great majority of insertional mutants can revert by precise cutting out of the transposon (Kleckner *et al.*, 1977). Clean excision is a rare event, however, imprecise excision being much more common (about 1000 times) and leading to some of the molecular rearrangements described in section e) above. A new phenomenon, nearly precise excision, has also been observed for Tn10 (Kleckner and Ross, 1978) which leads to the removal of all but 50 base pairs of the Tn10 insertion. In almost all cases, excision of Tn10 whether clean or imprecise does not lead to transposition to another site (Kleckner *et al.*, 1977), even if there is powerful selection for such an event (Foster, 1977). This contrasts with the findings for Tn1 (Hernalsteens *et al.*, 1977) and Tn7

(Results, this chapter). For Tn10, therefore, excision is probably not a stage in transposition. Bennett *et al.* (1977) compared the rate of loss of Tn1 with its transposition frequency to other replicons and concluded that transposition was at least 1000 times more common. This confirms that, in transposition, either the donor molecule normally retains the transposon or (according to the model of Berg, 1977) ceases to be a replicon.

h) Proteins involved in transposition. As mentioned earlier, there are no known proteins specified by insertion sequences. DNA sequencing of ISs [Ohtsubo and Ohtsubo, 1978 (IS1); Saedler and Ghosal, 1978 (IS2)] shows them to contain many inverted repetitions of varying sizes, including repeats within repeats. They are also AT rich, which may mean that certain regions melt relatively easily. Saedler and Ghosal, 1978, proposed a model for some IS2-associated phenomena based on selective melting and internal reduplication of defined regions on IS2 during replication of the carrier replicon. This involved no functions coded by IS2. Some of the characteristics of Tn10 can also be explained in terms of the physical properties conferred by its DNA sequence (Kleckner and Ross, 1978; Ross *et al.*, 1979) which may be recognised by enzymes coded by the host.

Direct evidence for the involvement of host functions in transposition and related events comes from the isolation of a chromosomal mutation affecting the behaviour of IS1 (Nevers *et al.*, 1977) and the identification of strains in which transposition is reduced (Results, this chapter). Also, a transposon has been created by bracketing *gal* genes in *E. coli* with directly repeated copies of IS1 (H. Saedler, personal communication reported in Calos *et al.*, 1978). This represents a possible route for the evolution of transposons and, since IS1 codes for no known proteins, suggests host functions must be involved in

transposition. However, how closely this construct resembles naturally occurring transposons has yet to be reported.

Work on Tn3 has shown that it was not formed merely by genes for β-lactamase production becoming flanked by structures analogous to insertion sequences (Heffron *et al.*, 1977; Gill *et al.*, 1978). Analysis of *in vitro* generated deletions of Tn3 showed that, in addition to the inverted repeats, there were regions within the transposon which were important for transposition. Complementation studies showed there is a *trans*-acting function which is essential for transposition. Another *trans*-acting function controls transposition negatively, thereby limiting its mutagenic effect. [A 19,000 dalton protein has been identified in minicells carrying Tn3 which corresponds to the product of this regulatory gene (Dougan *et al.*, manuscript in preparation)]. There is also a *cis*-acting function (or site) which is probably responsible for processing an intermediate* in transposition to give normal products. Tn3 is thus a highly evolved structure. Deletions in the central region of Tn7 also affect its ability to transpose (Results, this chapter).

i) Transposition at the molecular level. By sequencing the DNA at the junctions between IS1 and the surrounding chromosome it has been shown that IS1 insertion generates a directly repeated nine base pair sequence at each end of IS1. This sequence is different for each insertion studied and was originally present in a single copy on the recipient molecule. 18 of the 23 bases at the ends of IS1 are identical (but in inverted order) and there is no homology between these sequences

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*In the presumptive Tn3 transposition intermediates, the recipient replicon carries two copies of the transposon in the same orientation separated by a single copy of the complete genome of the donor replicon. Gill *et al.*, 1978, note the similarity between this structure and that seen in bacteriophage Mu-promoted integration of non-Mu DNA (Toussaint *et al.*, 1977). Similar structures have also been reported among the recipients of Tn1681 (So *et al.*, 1979) and Tn9 (J. Miller, personal communication reported in So *et al.*, 1979).

and the target site (Calos *et al.*, 1978; Grindley, 1978).

Similarly, 9 base-pair repeats have been observed flanking insertions of Tn5, Tn9, Tn903, Tn10 and IS4 (Kleckner and Ross, 1978; Sherratt, 1978; Oka *et al.*, 1978), and 5 base-pair repeats surrounding Tn1, Tn3, γδ, IS2 and bacteriophage Mu-1 insertions (Kleckner and Ross, 1978; Sherratt, 1978; Rosenberg *et al.*, 1978). The existence of these two classes may reflect the role of two different proteins each of which makes a different type of staggered nick in the recipient DNA strands. A model involving a single nick has also been proposed (Sherratt, 1978).

3. Role of transposable DNA *in vivo*

Transposable DNA sequences are potent agents for genetic change. By insertion or by causing deletions or rearrangements they can establish new linkages, mutate genes and whole operons and, at least in the case of IS2, can act as peripatetic promotors (Saedler *et al.*, 1974). Their prevalence in nature (Starlinger and Saedler, 1976; Kleckner, 1977) has led many authors to speculate on their role in the evolution of plasmids and chromosomes (Cohen, 1976; Nevers and Saedler, 1977; Saedler, 1977; Timmis *et al.*, 1979).

The work of Chow, 1977, detected many structures like insertion sequences present as inverted repetitions in the *E. coli* chromosome. These were not distributed randomly, most of the DNA segments bracketed by inverted repeats being approximately either 22 or 27.5 kilobases in length. As much as 18% of the *E. coli* chromosome may be organised in this way. This may be a requirement for the folding of the chromosome into a compact structure or it may reflect the modular nature of the chromosome which has evolved by the acquisition of transposable sequences from other organisms. The Lac transposon, Tn951, (Cornelis *et al.*, 1978) may have been such a module.

Where families of plasmids have been compared, or where plasmid spread has been studied *in vivo*, transposable DNA sequences are seen actively to have contributed to genetic versatility (Cohen, 1977; Datta *et al.*, 1979). Another example is where transposition allows the reversible amplification of genes conferring antibiotic resistance on plasmids in *Proteus mirabilis* and *Streptococcus faecalis* (Rownd *et al.*, 1975; Clewell and Yagi, 1977).

The importance of transposition is best demonstrated by considering the spread of antibiotic resistance. There is conclusive evidence for the world-wide distribution of TnA (Tn1, Tn2 and Tn3). Studies on enzymology and DNA homology have shown that TnA is present on a variety of different plasmids from widely separated sources (Matthews and Hedges, 1976; Heffron *et al.*, 1977a). Tn7 also seems to be in the process of spreading. It was originally observed as part of an IncI plasmid from *E. coli* (Barth *et al.*, 1976), and has since been found on other IncI plasmids in *E. coli* (Barth and Datta, 1977) and *Salmonella* (Richards *et al.*, 1978), and on an IncM plasmid in *Klebsiella aerogenes*, *E. coli* and *Citrobacter koseri* (Datta *et al.*, 1978). These are a few examples of how transposable genes can spread by becoming part of a transmissible plasmid. [For a treatise, see Richards, 1978]. IncP plasmids with their uniquely wide host range may be of particular importance in this process.

Transposable sequences can also spread by becoming part of phages [Jordan *et al.*, 1968; Shapiro, 1969 (ISs in λ); Berg *et al.*, 1975 (Tn5 and Tn6 in λ); Gottesman and Rosner, 1975 (Tn9 in P1); Botstein and Kleckner, 1977 (Tn10 in P22 and λ)] and even the single stranded phage fd can act as a vector for Tn1, Tn3 and Tn5 (Herrman *et al.*, 1978). Bacterial transposons can even enter plant cells via the oncogenic Ti plasmid found in *Agrobacterium tumefaciens* (Hernalsteens *et al.*, 1978).

Transposition is now being recognised as perhaps of more significance to the evolution of bacteria than the classical model where random mutations provide a basis for the action of natural selection. Plasmids and phages can carry transposons across wide phylogenetic divisions, and the activity of new genes introduced in this way can subsequently be modulated by other transposable sequences and events associated with transposons, as well as by host functions.

We are now close to an understanding of the mechanism of transpostion in bacteria. Further work may well show it to be of equal or greater importance in the evolution of higher organisms.

Results

1. Discovery of transposon 7 (Tn7, formerly TnC) as part of the IncIα plasmid, R483

Plasmids determining I pili form a compatibility complex (Hedges and Datta, 1973. See Jacob *et al.*, 1977a, for a list of IncI plasmids). Within the subgroup Iα, there is a hierarchy of compatibilities and one plasmid, R483, has anomalous properties (Hedges and Datta, 1973; Datta and Barth, 1975).

Multiply resistant strains of *E. coli* from calves that had been given large doses of trimethoprim and sulphonamide transferred trimethoprim (Tp) and streptomycin/spectinomycin (Sm) resistance to *E. coli* K12 separately from the other resistances (Hedges *et al.*, 1972). The plasmid in one such $(TpSm)^R$ isolate was designated R483. It was subsequently shown to code for the production of I pili (Datta and Hedges, 1973a) and colicin Ia (Datta and Barth, 1975).

Introduction of the Iα plasmids R144 or JR66a into a culture of *E. coli* K12 carrying R483 gave rise to clones which stably inherited the resistances of both plasmids in the absence of any antibiotic selection. The colicin production of R483 was eliminated, however, and normal transmissibility of $(TpSm)^R$ lost. Transfer was either greatly reduced or the TpSm resistances became linked to those of R144 or JR66a (Datta and Barth 1975; Barth *et al.*, 1976). This behaviour was analogous to that shown by the ampicillin resistance genes from RP4 which can transpose to the bacterial chromosome and other plasmids (Richmond and Sykes, 1972; Hedges and Jacob, 1974).

From the "doubles" stably carrying the resistances of R483 and R144 or JR66a, strains were obtained which were resistant only to TpSm. In these strains, the resistances were entirely non-transmissible and no plasmid DNA could be detected (Barth *et al.*, 1976). This strongly

suggested that the resistance genes of R483 had become inserted in the bacterial chromosome, as had been proposed earlier (Datta and Hedges, 1973a).

2. Mapping of the TpSm resistances from R483 in the *E. coli* chromosome

J62-TnC1 and J62-TnC2 (Barth *et al.*, 1976) are two independent isolates of strain J62 carrying the TpSm resistances from R483 in a non-transmissible form, probably inserted in the chromosome. Preliminary mapping was done by introducing F*lac* (Low, 1972) into the strains and then crossing them with the multiply-marked recipient, AB1157. Broth matings were carried out for 90 minutes before plating the mixture onto medium containing trimethoprim plus the nutritional requirements of AB1157. Colonies were purified on the same medium and tested for the co-inheritance of chromosomal markers. The results are shown in Table 4.2. If the frequency of co-inheritance is taken as a measure of the closeness of two markers, then for both J62-TnC1 and J62-TnC2, the TpSm resistance genes behave as if they were inserted in the chromosome between *argE* and *mtl* [i.e. between 88 and 80 minutes on the *E. coli* K12 map (Bachman *et al.*, 1976)]. The Sm^R marker was always co-mobilised with Tp^R.

The site of insertion was more precisely determined by P1 transduction. Lysates were prepared from J62-TnC1 and J62-TnC2, titrated, and used to transduce strain CRT46 which has two mutations in the 80-88 minute region of the chromosome (*dnaA* (t.s.) at 81.8 minutes and *ilv* at 83.2 minutes). The results are shown in Table 4.3, lines 1-8. Both donor strains behaved indistinguishably. Trimethoprim resistance was co-transducible with both *ilv* and *dnaA* at high frequency suggesting that transposition had taken place to a site between these two markers, probably nearer to *dnaA*. This was confirmed by comparing the co-transduction frequencies of *dnaA* and *ilv* before and after the insertion

Table 4.2. Mobilisation of Tp^R from J62-TnC1 and J62-TnC2 to AB1157 by F'*lac*: frequency of co-inheritance of chromosomal markers

Unselected marker		Cotransfer with Tp^R from:	
Gene	Map position (min)	J62-TnC1	J62-TnC2
leu$^+$	2	24(34)[1]	20(28)
argE$^+$	88	39(55)	37(52)
mtl$^+$	80	23(32)	32(45)
galK$^+$	17	0(0)	0(0)
Sm^R	?	71(100)	71(100)

71 Tp^R colonies from each cross were purified and tested for the presence of other markers.

1 Figures in brackets are percentages.

Table 4.3. P1 cotransduction frequencies of markers from J62, J62-TnC1 and J62-TnC2 into CRT46 (*ilv dnaA*(t.s))

P1 donor	Selected marker[1]	Unselected marker	Cotransduction frequency[2]	Mapping distance (min)[3]
J62-TnC1	Tp^R	Ilv^+	30/112(26.8)	0.71
J62-TnC2	Tp^R	Ilv^+	26/115(22.6)	0.78
J62-TnC1	Ilv^+	Tp^R	46/112(41.1)	0.51
J62-TnC2	Ilv^+	Tp^R	50/110(45.5)	0.46
J62-TnC1	Tp^R	$DnaA^+$	78/114(68.4)	0.24
J62-TnC2	Tp^R	$DnaA^+$	75/115(65.2)	0.27
J62-TnC1	$DnaA^+$	Tp^R	76/111(68.5)	0.24
J62-TnC2	$DnaA^+$	Tp^R	44/71(62.0)	0.29
J62	Ilv^+	$DnaA^+$	78/408(19.1)	0.85
J62-TnC1	Ilv^+	$DnaA^+$	22/407(5.4)	1.24
J62-TnC2	Ilv^+	$DnaA^+$	32/609(5.3)	1.25
J62	$DnaA^+$	Ilv^+	55/409(13.4)	0.98
J62-TnC1	$DnaA^+$	Ilv^+	6/245(2.4)	1.42
J62-TnC2	$DnaA^+$	Ilv^+	8/323(2.5)	1.42

1 All selections were on minimal medium. $DnaA^+$ colonies were selected by their ability to grow at 42°C, all other selections being done at 30°C. Purified clones were tested for the unselected marker. Transduction frequency was typically 10^{-5} per p.f.u. for all markers.

2 Figures in brackets are percentages.

3 These were calculated according to Bachman *et al.*, 1976, using an equation derived by Wu, 1966.

of the transposon (Table 4.3, lines 9-14). In either direction, there was a marked drop in co-transduction frequency after insertion of the drug resistance genes, showing that the transposon was located between *dnaA* and *ilv* on the chromosome.

Using a formula developed by Wu, 1966, which relates P1 co-transduction frequency to the physical separation of two markers, it is possible to estimate the size of the inserted DNA. The relationship is:

$$\text{Distance between two markers} = \left(\text{Effective size of P1 genome}\right)\left(1 - \sqrt[3]{\text{co-transduction frequency}}\right)$$

Taking the effective size of the P1 genome as 2 minutes (Bachman *et al.*, 1976) and averaging the co-transduction frequencies for reciprocal crosses, this gives:

dnaA ——— *ilv* distance of 0.91 minutes (for J62)
dnaA - TpSm - *ilv* distance of 1.30 minutes (for both J62-TnC1 and J62-TnC2)

The transposon is therefore approximately 0.39 minutes or 9.8 Md.*
This is in good agreement with the value of 9.0 obtained by electron microscopy (M. van Montagu, personal communication).

The two independent events which produced J62-TnC1 and J62-TnC2 resulted in the insertion of similar sized DNA segments at apparently the same chromosomal site. To confirm the existence of a highly preferred site of integration, three further, independent examples of Tn7 insertion into the chromosome (J53-TnC3-5, Table 4.4) were used as P1 transductional donors. Ilv^+ and Tp^R were co-transducible from all three strains at comparable frequencies, proving that the region around *ilv* is a hot-spot for Tn7 insertion.

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*This was calculated assuming chromosomal transfer to take 100 minutes (Bachman *et al.*, 1976) and the molecular weight of the chromosome is 2500×10^6 daltons (Cooper and Helmstetter, 1968).

Table 4.4. Pl cotransduction frequencies of markers from chromosomally transposed strains to AB1450(*ilv*)

Pl donor	Selected marker[1]	Unselected marker	Cotransduction frequency[2]
J53-TnC3	Tp^R	Ilv^+	32/76(42)
J53-TnC3	Ilv^+	Tp^R	15/49(31)
J53-TnC4	Tp^R	Ilv^+	22/65(34)
J53-TnC4	Ilv^+	Tp^R	4/13(31)
J53-TnC5	Tp^R	Ilv^+	38/78(49)
J53-TnC5	Ilv^+	Tp^R	21/52(40)

1 All selections were on minimal medium. Ilv^+ or Tp^R colonies were purified selectively and tested for the co-inheritance of the unselected marker. Transduction frequencies were typically 10^{-5} per p.f.u. for both markers.

2 Figures in brackets are percentages.

3. Measurement of the frequency of transposition

Strain W3110T⁻-TnC (Barth and Grinter, 1977) which carries a chromosomally inserted copy of Tn7 was infected with RP4. A purified single colony of (RP4) W3110T⁻-TnC was then used to estimate the frequency of transposition of Tn7 to RP4 by both direct and indirect measurements. A broth culture of (RP4) W3110T⁻-TnC was mixed with J53 for approximately 16 hours before plating serial dilutions on minimal medium containing the nutritional requirements of J53 plus either trimethoprim (10 µg/ml) or ampicillin (100 µg/ml). After incubation, an average of 238 colonies appeared on the ampicillin plates at a dilution of 10^{-5}, compared with 95 on trimethoprim at a 10^{-2} dilution. Taking these colonies to represent the number of RP4 and RP4-Tn7 transfers respectively, the frequency of transposition was $95 \times 10^{2}/238 \times 10^{5}$, or 4×10^{-4} per RP4 transfer. This is an indirect measurement of transposition frequency. A direct measure was achieved by replica plating ampicillin resistant colonies from the 10^{-4} dilution onto medium containing trimethoprim. Out of an estimated 23,800 Ap^{R} colonies, 14 were also Tp^{R}, equivalent to a transposition frequency of 6×10^{-4} per RP4 transfer.

Thus, by both methods, transposition frequency was about 5×10^{-4} per RP4 transfer. The RP4 in the donor strain had been freshly introduced. Retesting the transposition frequency after several subculturings of the donor gave no significant change in measured transposition frequency, suggesting that an equilibrium between RP4 and RP4-Tn7 plasmids is rapidly established in the population.

4. Attempts to introduce Tn7 into R300B

R300B is a small (5.7 Md) non-conjugative plasmid conferring resistance to streptomycin (Sm) and sulphonamide (Su). It was originally isolated accompanied by a large (57 Md) mobilising plasmid

(R300A) which confers no antibiotic resistances (see Barth and Grinter, 1974). It was of interest to make a Tn7 derivative of a small plasmid since this would facilitate characterisation of Tn7 by heteroduplex and restriction enzyme analysis. Three different techniques were used in an attempt to isolate Tn7 as part of R300B:

a. Mobilisation - Strain J62-TnC1(R300A)(R300B) was constructed and crossed with J53, selecting for the transfer both of Tp^R and the high level of Sm^R conferred by R300B. Both resistances readily transferred to J53 (frequency 10^{-3}-10^{-4} per donor per hour), but Tp^R was always associated with R300A, i.e. it was readily re-transferred without the SmSu resistance of R300B.

b. Transduction - P1 lysates were prepared from J62-TnC1(R300A)(R300B) and J62(R483)(R300B). The transductional recipient was J53 and selection was for Tp^R, high-level Sm^R, or Tp^R + high level Sm^R. Both markers were readily transducible (10^{-5} per p.f.u.) but no co-transduction was seen.

c. Transformation - Plasmid DNA was prepared from J62-TnC1(R300B) and used to transform *E. coli* C, selecting either for Sm^R or Tp^R. Roughly 10^5 transformants were obtained on Sm, but none on Tp. This last observation ruled out the possibility that Tn7 preferentially inserted into the Sm^R genes of R300B, which might have explained the results in a and b.

It was concluded that Tn7 does not readily transpose to R300B.

5. Ability of Tn7-generated mutants to revert

Screening of Tn7 derivatives of RP4 isolated as described in section 3 above showed that about 4% were Km^S, Tc^S or Tra^-. That these mutations were the result of Tn7 insertion was proved by showing that Tn7 insertions giving a particular phenotypic change all clusted in one region on the RP4 genetic map (Barth and Grinter, 1977). All

Km^S, Tc^S and Tra^- derivatives of RP4 were tested for their ability to revert as this would indicate that the mutated genes had not been damaged by Tn7 insertion.

10 ml cultures of the mutants in J53 were grown to late log phase (c. 10^9 cells/ml) in broth and concentrated tenfold by centrifugation. The Tra^- cultures were mixed with equal volumes of similarly treated C600 and left for 3 hours at 37°C before plating on minimal medium selective for Km^R C600. The Km^S or Tc^S cultures were plated on minimal medium for J53 containing Km (25 µg/ml) or Tc (10 µg/ml).

None of the 12 strains with no detectable transfer described by Barth *et al.*, 1978, gave Tra^+ revertants. The level of detection was only 10^{-7}-10^{-8}, however, even assuming any revertants would have the normal RP4 transfer frequency of c. 10^{-3} per donor per hour. Reversion at a high rate (10^{-6}, say) to a phenotype conferring 10% of the normal transfer frequency would not be detected.

The Km^S and Tc^S derivatives tested for reversion were those described in Barth *et al.*, 1978. In addition, two as yet unmapped Tc^S derivatives, pRP85 and 86, were also screened. The results are given in Table 4.5.

Of the fifteen strains tested, only three gave drug resistant revertants at a detectable frequency (i.e. > 10^{-10}). Several differences are apparent between the revertible mutants when the reversion frequencies and nature of the revertants are compared. pRP83(Km^S) reverted to confer Km^R at a rate (10^{-7}) between 10^2 and 10^3 times higher than that of the other two revertible mutants (pRP84 and 88, Km^S and Tc^S respectively). Also, revertants of pRP83(pRP831-pRP836) and pRP88(pRP881-pRP882) retained the $Tp^R Sm^R$ of Tn7 in addition to the markers of RP4. Of the four revertants of pRP84(pRP841-pRP844), however, only one (pRP841) conferred this phenotype, the remainder

Table 4.5. Reversion of Tn7 derivatives of RP4 conferring either Km^S or Tc^S

Plasmid[1]	Phenotype	Reversion frequency	Resistance pattern of revertants	Resistance pattern transferred by revertants
pRP33	Km^S	$< 10^{-10}$	-	-
pRP48	"	$< 10^{-10}$	-	-
pRP65	"	$< 10^{-10}$	-	-
pRP83	"	$c\ 10^{-7}$	$(ApTcKmTpSm)^R$-pRP831-836	$(ApTcKmTpSm)^R$
pRP84	"	4×10^{-10}	$(ApTcKmTpSm)^R$-pRP841 $(ApTcKm)^R$-pRP842-844	$(ApTcKm)^R$ $(ApTcKm)^R$
pRP25	Tc^S	$< 10^{-10}$	-	-
pRP64	"	$< 10^{-10}$	-	-
pRP70	"	$< 10^{-10}$	-	-
pRP71	"	$< 10^{-10}$	-	-
pRP72	"	$< 10^{-10}$	-	-
pRP73	"	$< 10^{-10}$	-	-
pRP85	"	$< 10^{-10}$	-	-
pRP86	"	$< 10^{-10}$	-	-
pRP87	"	$< 10^{-10}$	-	-
pRP88	"	2×10^{-10}	$(ApTcKmTpSm)^R$-pRP881 & 882	$(ApTcKmTpSm)^R$

Reversion frequency was measured as described in the text. Drug resistant colonies were purified on selective medium and their resistance pattern checked with Multodiscs (Oxoid). They were subsequently transferred to W3110T$^-$ by plate mating, selecting for ampicillin transfer, purified selectively, and retested for drug resistance pattern by Multodisc and ditch tests.

1 These are described in Barth and Grinter, 1977 and Barth *et al.*, 1978, except for pRP85 and 86 (see text).

Molecular weights of the Km^S and Tc^S Tn7 derivatives of RP4

Plasmid	Molecular weight (Md.)
pRP33	42.1
pRP48	44.1
pRP65	44.1
pRP83	44.6
pRP84	43.6
pRP25	43.0
pRP64	43.6
pRP70	43.9
pRP71	44.1
pRP72	44.2
pRP73	44.4
pRP85	?
pRP86	?
pRP87	42.7
pRP88	44.5

Data from Barth and Grinter, 1977 and Barth *et al*, 1978.

conferring $(ATK)^R$ only.

To check that reversion was associated with a change in the plasmid rather than a host mutation, all the revertant clones were plate-mated with W3110T$^-$, selecting for ampicillin resistance transfer. Exconjugants were purified and retested for their antibiotic resistance pattern. All the revertants of pRP83 and pRP88 transferred all their resistances together, whereas pRP841 failed to transfer the markers of Tn7. This is consistent with the revertants of pRP83 and pRP88 having undergone an internal transposition, Tn7 moving to a new site on the molecule and thereby relieving its effect on the antibiotic resistance gene near or in which it was originally inserted. Revertants of pRP84, on the other hand, have all lost the transposon from the plasmid, although in the case of pRP841 it has been retained by the chromosome. This was confirmed directly. The site of Tn7 insertion in pRP831 was mapped using restriction enzyme digestion and sucrose gradient analysis of the fragments generated. Tn7 was found to have shifted from its site at 23 Md on the RP4 map in pRP83 to a new position at 32 Md in pRP831 (Barth and Grinter, 1978). Also, pRP841 was shown to be indistinguishable from RP4 (Barth and Grinter, unpublished observations).

The significance of these results is discussed later, together with the finding that the ability of these strains to revert to a drug resistant phenotype disappeared after repeated subculturing or storage in stab.

6. Properties of Tn7 derivatives with deletions generated *in vitro*

In a series of experiments to locate essential regions of RP4, restriction fragments were excised from Tn7 derivatives of RP4 (Barth and Grinter, 1977 and unpublished data). Several plasmids were thus created in which Tn7 had suffered deletions or rearrangements, and these are shown in Figure 4.1.

Figure 4.1: Restriction maps of Tn7 and derivatives which have deletions

Tn7 is shown at the top with vertical lines indicating cut sites for restriction enzymes *Eco*R1(E), *Hind*III(H) and *Bam*H1(B). The co-ordinates of these sites are shown in Md. on the next line. The various deletions are shown by dotted lines and the names of the plasmids carrying them are given on the right hand side together with their conferred phenotypes. The $(TpSm)^R$ determinants lie to the left of the *Eco*R1 site, in the first 2.7 Md. The map of Tn7 is from Richards, 1978.

1 pRP221, 222 and 311 all have the same segment missing from Tn7. In pRP221 (see Barth and Grinter, 1977) the two ends of the transposon are separated and the orientation of the right hand end reversed. In pRP222 the ends have been brought back together in their normal orientation. pRP311 is like pRP221 except the ends are not so widely separated.

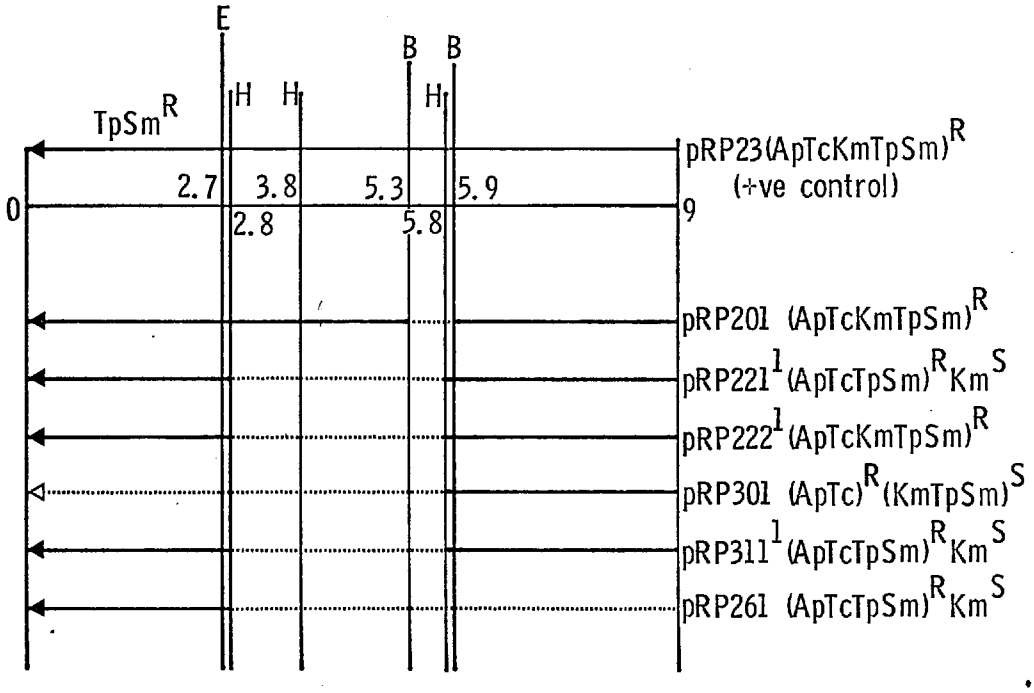


Figure 4.1 : Restriction maps of Tn7
and derivatives which have deletions.

The derivatives of Tn7 were tested for their ability to transpose (except for pRP301 which is $Tp^S Sm^S$). The method was to introduce a plasmid incompatible with RP4 and look for stable retention of Tp^R; if found it would indicate that the Tp^R was transposed from its RP4 vector. pRP301 could not be so tested; instead it was tested for ability to accept a new copy of Tn7.

Each plasmid with its Tn7-deletion was transferred to W3110*recA* (HH27) by plate mating, selecting for Tc^R. pRP23 was included as a positive control. (pRP301 was transferred to W3110*recA*-Tn7. RP4 acted as a positive control in this case). Transconjugant clones were purified selectively, their drug resistance pattern checked by Multodisk, and their U.V. sensitivity compared with W3110T$^-$ and W3110*recA*.

(pRP301)W3110*recA*-Tn7 was crossed with J53-2, selecting for transfer of either Tp^R or Tc^R. It behaved indistinguishably from (RP4)W3110*recA*-Tn7 in that Tp^R was transferred to J53-2 at a frequency of 10^{-6} per donor per hour, and there was roughly one Tp^R transconjugant for every 10^3 which were Tc^R. Thus pRP301 is as good a recipient of Tn7 as RP4, and hence the presence of the tail end of Tn7 carried by pRP301 (from 5.8-9.0 Md, Fig. 4.1) does not inhibit transposition.

R1033(IncP) was transferred into the W3110*recA* isolates carrying the other derivatives of Tn7, selecting for the transfer of chloramphenicol resistance (Cm^R) and simultaneously for the retention of Tp^R from Tn7. Transconjugants were purified on this double selection and then repurified twice more on drug-free medium to allow incompatible plasmids to segregate. 20 colonies from each cross were then tested for resistance to Cm and Tp. The results are shown in Table 4.6(a).

Apart from the control, pRP23, only pRP201 and pRP311 gave colonies resistant to both Cm and Tp (1/20 in both cases). These doubly resistant colonies and one from the pRP23 control were purified

Table 4.6 (a). Resistance patterns observed after release of double selection

Plasmid double in W3110 *recA*	Resistance patterns after non-selective purifications Tp^RCm^R	Tp^SCm^R	Tp^RCm^S
(pRP23)(R1033) +ve control	20/20	0/20	0/20
(pRP201)(R1033)	1/20	14/20	5/20
(pRP221)(R1033)	0/20	10/20	10/20
(pRP222)(R1033)	0/20	20/20	0/20
(pRP311)(R1033)	1/20	17/20	2/20
(pRP261)(R1033)	0/20	13/20	7/20

Strains containing R1033 and the incompatible Tn7 derivative of RP4 were constructed as described in the text. Selection for both plasmids was maintained during a purification step and then released for two subsequent purifications. Twenty colonies from each purification were then tested on antibiotic ditch plates.

Table 4.6 (b). Resistance patterns after further selective and non-selective growth

Plasmid double in W3110 *recA*	Resistance pattern after growth on: chloramphenicol	trimethoprim	nutrient medium, no selection
(pRP23)(R1033) +ve control	4/4 Tp^RCm^R	4/4 Tp^RCm^R	4/4 Tp^RCm^R
(pRP201)(R1033)	4/4 $Tp^S_RCm^R$	4/4 $Tp^R_RCm^S$	4/4 $Tp^R_RCm^S$
(pRP311)(R1033)	4/4 Tp^RCm^R	4/4 Tp^RCm^R	4/4 Tp^RCm^R

The $(TpCm)^R$ colony from (pRP201)(R1033) and that from (pRP311)(R1033) - see Table 4.6 (a) - were purified on Cm, Tp or nutrient medium without selection. A colony from the control was also included. 4 colonies from each purification were ditch-tested as above.

again on lysed blood medium containing Cm or Tp, or on drug-free nutrient agar. Colonies from each purification were then re-tested for Cm^R and Tp^R. The results are shown in Table 4.6(b). As expected, the control was stably resistant to both antibiotics, indicating that Tn7 had transposed either to R1033 or the bacterial chromosome. All colonies derived from the one originally harbouring R1033 and the Tp^R from pRP201, however, have now lost either Cm^R or Tp^R, showing that the resistances were present on separate plasmids and that transposition had not taken place. In contrast, there was no segregation of resistances observed after purification of the colony carrying R1033 and the Tp^R from pRP311. Transposition thus appeared to have taken place.

Three colonies in which "transposition" from pRP311 had occurred were tested and found to be resistant to ApTcCmGmSuTpSm i.e. they had all the resistances conferred by pRP311 and R1033 except that they were Km^S. Six colonies were also checked for U.V. sensitivity. All were sensitive. These colonies all transferred their resistances *en bloc*, regardless of whether selection was initially for Cm^R or Tp^R. It therefore appears that transposition of $TpSm^R$ has taken place at low frequency from pRP311 to the Km^R gene of R1033. This is an unexpected result because pRP221 and 222 have both lost the same segment of Tn7 as pRP311 and neither transposes.

Thus, in the absence of molecular analysis, the nature of the $Tp^RSm^RKm^S$ derivative of R1033 remains indeterminate. It could be the result of a bizarre, *recA* independent fusion between pRP311 and R1033.

None of the other deletion derivatives of Tn7 transposed. pRP261 lacks the right-hand end of the transposon, but pRP201, 221 and 222 have lost material only from the central region of the transposon showing that, as with Tn3, (Gill *et al.*, 1978) it codes for functions necessary

for transposition. Also, the observation that pRP301 can readily accept a further copy of Tn7 shows that any genes which may prevent two copies of Tn7 entering the same plasmid (analogous to TnA, Robinson *et al.*, 1977) are not present in the region from 5.8 Md to the right-hand end of the transposon.

7. The importance of host-coded functions in transposition

It was originally reported that TnA(Tn1) transposition was *recA* dependent (Hedges and Jacob, 1974). Subsequent work (Heffron *et al.*, 1977) showed this was not the case. Repeating the earlier work, however, confirmed that Tn1 transposition was undetectable in the *recA* strain AB2463 (R. W. Hedges, unpublished data), suggesting that a mutation in this strain prevented transposition. The following experiment was carried out to test the effect of the host strain on Tn7 transposition.

P1 lysates were prepared from (RP4)J62 and J62-TnC1 and used to transduce several different host strains (see Table 4.7). Selection was for Tc^R(RP4) or Tp^R(Tn7 from J62-TnC1). The lysate from the plasmid-bearing strain was included to show that all the recipients were P1 transducible.

The results show that all the recipients were readily transduced by the lysate from (RP4)J62 at roughly the same frequency (Table 4.7). There were wide variations in the abilities of these strains to act as recipients for Tn7, however. In AB1157(*recA*$^+$ AB2463), trimethoprim resistant transductants were readily detectable, but at a much reduced frequency as compared with W3110T$^-$. They are probably due to homologous recombination because in the *recA* derivatives of AB1157 no Tp^R transductants were detected. In contrast, Tp^R transductants were readily detectable in the other *recA* strain, AB1553.

The results show that transposition is reduced by a factor of

Table 4.7. Pl transduction of RP4 and Tn7 to various recipients

Pl donor	Recipient	Relevant genotype	Selected marker	Transduction frequency per p.f.u. x 10^7
(RP4)J62	W3110T$^-$	*recA*$^+$	Tc^R	30
J62-TnCl	W3110T$^-$	*recA*$^+$	Tp^R	540
(RP4)J62	JC1553	*recA*	Tc^R	10
J62-TnCl	JC1553	*recA*	Tp^R	10
(RP4)J62	AB1157	AB2463*recA*$^+$	Tc^R	58
J62-TnCl	AB1157	AB2463*recA*$^+$	Tp^R	27
(RP4)J62	AB2463	*recA*	Tc^R	10
J62-TnCl	AB2463	*recA*	Tp^R	< 0.03
(RP4)J62	AB2487	AB2463 *thy*	Tc^R	30
J62-TnCl	AB2487	AB2463 *thy*	Tp^R	< 0.03

The transductions were at a multiplicity of infection between 0.1 and 0.2. Selection was on minimal medium containing the relevant growth requirements plus Tc(10 µg/ml) or Tp(10 µg/ml except for W3110T$^-$ and AB2487 which both require thymine - a trimethoprim antagonist - and therefore 100 µg/ml of Tp was used). Controls omitting the phage were carried out and gave no colonies in all cases.

more than 100 in strains AB2463 and AB2487, proving that host functions are involved in Tn7 transposition.

Summary of Results

1. Tn7 was shown to have a unique or highly preferred site of insertion between *dnaA* and *ilv* on the *E. coli* K12 chromosome. The size of the inserted DNA was about 9.8 Md.

2. The frequency of Tn7 transposition from the *E. coli* chromosome to RP4 was 5×10^{-4}.

3. Tn7 does not readily transpose to R300B.

4. Most mutants caused by Tn7 insertion do not revert at detectable frequencies.

5. The central region of Tn7 codes for functions essential for transposition.

6. It was shown that host functions are necessary for Tn7 transposition.

Discussion

Specificity of Tn7 insertion in the chromosome. The precise site of insertion cannot be determined from the data in Table 4.3 for several reasons:

1. Reciprocal crosses involving Tp^R and Ilv^+ give co-transuction frequencies differing by 14%. The reason for this may be that packaging of chromosomal DNA by P1 is initiated at specific sites. This question is discussed by Bachman *et al.*, 1976, and this thesis, Chapter 6.

2. It is not known if selection for trimethoprim resistance demands the inclusion of the complete transposon in a transducing particle. If so, this would bias any estimate of the site of insertion towards the gene (*dnaA* or *ilv*) to which the transposon was nearer.

3. If the entire transposon is carried by a transducing particle, when introduced into a new host it may well insert into the recipient's chromosome more efficiently than any chromosomal genes carried on the same DNA fragment.

These factors complicate interpretation of the transduction data. However, the site of Tn7 insertion is definitely between *dnaA* and *ilv* and it is within this region that the origin of chromosomal replication is now believed to be (Fayet and Louarn,1977; Marsh, 1978).

It is unlikely that Tn7 has a requirement for a specific sequence at the site of insertion because it inserts into RP4 at at least 40 sites (Barth and Grinter, 1977; Barth *et al.*, 1978). Tn7 inserts into the chromosome very readily indeed, (Datta and Hedges, 1973a; Hedges and Datta, 1973; Datta and Barth, 1976) and it could be that, analogous to λ, it has an "attachment site" at which it preferentially inserts but, in the absence of this site, it can insert at a reduced frequency at many other sites (Enquist and Weisberg, 1977). Another possibility is that Tn7 inserts near the origin of replication because

this region is present in most copies per cell at any given time. If this is true for Tn7, however, it has not been shown to be true for any other transposon.

Two experiments might clarify the specificity of Tn7 insertion. Firstly, the site of insertion could be precisely mapped by heteroduplex analysis of Tn7 derivatives of F's carrying the chromosomal origin. Secondly, the specificity of insertion could be studied in integratively suppressed strains, the chromosomes of which are replicating from an origin on a plasmid inserted at a known site. This could be either in the presence or absence (by deletion) of the normal chromosomal origin.

Specificity of Tn7 insertion into plasmids. For any transposon to insert into a plasmid, there must be suitable and accessible sites on that plasmid, and insertion must not inactivate any essential plasmid functions. Tn7 insertion into RP4 is not random and most regions in which insertions have not been detected probably contain essential genes (Barth and Grinter, 1977 and unpublished data). The exception to this is the Tn1 region of RP4. No Tn7 insertions have been detected here whereas for Tn501 there seem to be hot spots for insertion within this region (Grinstead *et al.*, 1978). Clearly different transposons have different specificities.

The failure to obtain Tn7 derivatives of R300B is probably due to lack of suitable sites on the plasmid. Tn3 transposes readily to RSF1010, a plasmid indistinguishable from R300B, at a minimum of 20 sites (Heffron *et al.*, 1977a) and R300B can accept two transposons determining Km^R (Tn5 and Tn711, Richards, 1978). Therefore, R300B can act as a transposon recipient. Failure of Tn7 to transpose to R300B might be thought due to the large size of the transposon (9 Md) in relation to the plasmid (5.7 Md). However, Heffron *et al.*, 1977b, have obtained Tn3(3.2 Md) derivatives of plasmid pMB8 (1.8 Md).

Whatever determines the specificity of insertion will probably only be revealed by DNA sequencing of large numbers of insertion sites. Where such work has been undertaken for IS1 (Grindley, 1978; Calos *et al.*, 1978), no obvious relationship was discovered between the different insertion sites, although insertion may be at some distance from the recognition site. Alternatively, it may be a feature of the three dimensional structure of the DNA which is recognised, rather than any particular sequence.

Reversion of antibiotic sensitive Tn7 derivatives of RP4. 12/15 Km^S or Tc^S RP4-Tn7 plasmids gave no reversion to a drug resistant phenotype at detectable frequency ($> 10^{-10}$) [Table 4.5]. Failure to revert may indicate that the inactivated gene has been damaged in some way by the insertion and this is consistent with the observation that the vast majority of Tn7 derivatives of RP4 have a molecular weight less than the expected 45 Md (RP4 - 36 Md + Tn7 - 9 Md). Of 47 plasmids examined, only 3 had a molecular weight $\geq$ 45 Md. The remainder had suffered deletions, detectable by sucrose gradient analysis, of up to 3 Md (Barth and Grinter, 1977; Barth *et al.*, 1978). In one case, the deletion has been shown to be at the site of insertion, and to involve only RP4 DNA (G. Engler, M. van Montagu and J. Schell, personal communication). Consequently, those Tc^S and Km^S plasmids which do not revert may have undergone such a deletion.

The three clones which were capable of reversion could either have suffered no deletion on insertion of Tn7 into the affected gene, or be polar mutations resulting from an insertion adjacent to but not in the drug resistance gene. Reversion in the former case requires precise excision of the transposon. In the latter case, polarity would be relieved by any deletion which removed signals on Tn7 terminating transcription. pRP83 which reverted at high frequency compared

to the other two revertible mutants may have been such a polar mutation.

Reversion could be accompanied by loss of the transposon (pRP842-844) or by its moving to another site on RP4 (pRP831). [It is not possible to say in the one case (pRP841) where Tn7 was retained by the chromosome whether deletion of the transposon was accompanied by transposition to the chromosome or if this had occurred beforehand.] Molecular analysis of one plasmid which had lost Tn7(pRP841) showed it to be indistinguishable from RP4.

Precise and imprecise excision have been reported for Tn10, but neither event leads to transposition elsewhere (Botstein and Kleckner, 1977). In contrast, polar mutations caused by Tn1 can revert, often accompanied by transposition to a new site on the plasmid (Hernalsteens *et al.*, 1977). Tn7 excision, therefore, more closely resembles that of Tn1 and is not necessarily accompained by the loss of the donor replicon.

Reversion was at a much lower frequency than acquision of Tn7 by RP4 and thus the excision of Tn7 which seems to accompany reversion is probably not a stage in normal transposition.

The loss of revertability by pRP83, 84 and 88 after subculturing or storage may be due to deletions being formed in RP4 after the insertion of Tn7. In this respect Tn7 resembles Tn10 which can generate a variety of post-insertional deletions (Ross *et al.*, 1979). The distinction between deletions formed on insertion or as a subsequent event is not a clear one. Tn10 insertions causing gene inactivation are almost always revertible, showing that no material is deleted from the recipient as a consequence of insertion (Foster, 1977). Post-insertional deletions can be detected at a frequency of 10^{-3}-10^{-4}, however (Ross *et al.*, 1979). The behaviour of Tn7 could be explained if its insertion was "clean", like Tn10, but subsequently deletions

were formed at a much higher frequency.

Mechanism of transposition. Apart from its unique or highly preferred chromosomal site, Tn7 has no known properties not shown by other transposalbe sequences. As suggested above, its specificity of insertion into the chromosome may be analogous to λ attachment. Its specificity of insertion into other plasmids may also be characteristic, although insufficient data are available. In its remaining properties, Tn7 differs only qualitatively from other transposons. However, no transposon shares all the other properties of Tn7, and no transposons conferring distinguishable phenotypes have been shown to behave identically, suggesting that different mechanisms are operating.

Many of the properties of transposons may depend upon the nature of the repeated DNA sequences at their ends. Several distinguishable types have been reported (see Kleckner, 1977). Also, differences between transposon-coded properties (Gill *et al.*, 1978) may affect the frequency and specificity etc. of transposition as well as determining a variety of transposition mechanisms.

The role of the repeated sequences at the ends of transposons could be determined by bracketing identifiable but non-transposing genes with insertion sequences or the ends of a transposon. *In vivo* or *in vitro* techniques could be employed and the constructs compared with naturally occurring transposons. H. Saedler has already begun such work (Calos *et al.*, 1978).

Proteins encoded by Tn3 have been identified in minicells and their functions studied by complementation between deletion mutants mapping at different sites in the central region of the transposon (Dougan *et al.*, manuscript in preparation; Gill *et al.*, 1978). This work could be extended to other transposons, and complementation between different transposons studied.

The complementation studies have already identified a *trans*-acting repressor of Tn3 transposition, and Robinson *et al.* have identified a *cis*-acting function which prevents a plasmid acquiring more than one copy of the same transposon. If such functions are present on all transposons, they could be put into groups having common susceptibility to these functions.

Finally, the role of host functions in transposition and related events has to be elucidated. Nevers and Saedler, 1977, found a mutation affecting IS1 deletion formation (but not excision), and a chromosomal mutation affects transposition by Tn1 and Tn7 (this chapter). These mutations must be mapped, their effects on other transposable sequences studied, and other mutants isolated in order to characterise the role of the host in transposition.

CHAPTER 5

PLASMIDS AS VECTORS FOR CHROMOSOMAL GENES

Abstract

In this chapter the properties of recombinant plasmids generated *in vivo* and *in vitro* are examined. Hybrid plasmids were generated by the *in vitro* insertion of a particular restriction fragment of bacteriophage λ DNA into the *Hind*III cut site on RP4. In some of these plasmids the inserted λ DNA also carries the insertion sequence IS1. Comparison of the chromosomal mobilisation frequencies of these plasmids in different genetic backgrounds allowed the relative contributions of IS1, the *recA* gene and extensive DNA homology with the bacterial chromosome to be studied. An R' generated *in vivo* was also characterised. This plasmid carries *arg* genes from the *Pseudomonas* chromosome which are expressed in *E. coli*. Using P1 transduction, the site of insertion of *Pseudomonas* DNA on the plasmid was localised and the existence of a *Pseudomonas* regulatory gene inferred.

Introduction

The F (fertility) factor was discovered through its ability to mobilise the *E. coli* chromosome (Lederberg and Tatum, 1946). Subsequent characterisation (Hayes, 1953) showed it to be an autonomous replicon which was transmissible at high frequency, transfer of the chromosome being a much rarer event associated with carriage of F. It became clear that F could exist in a variety of interchangeable states; either autonomous (F^+), integrated into the chromosome (Hfr), or, after imprecise excision from the Hfr state, as an F' (F-prime) carrying some chromosomal genes. Each state has its own characteristic mode of chromosome transfer, and all have been invaluable in constructing the genetic map of *E. coli* (for reviews, see Low, 1972 and Bachman *et al.*, 1976).

In the F^+ state, chromosomal markers are transferred quite randomly at a uniformly low (10^{-6} per donor) frequency, while an Hfr transfers genes in a fixed order from a particular origin and with a gradient of transmission frequencies from 10^0 to 10^{-6} depending on the nearness of the gene to the origin of transfer (Jacob and Wollman, 1961). The order of gene transfer is constant for any one Hfr, but many Hfr strains are known having differing origins and orders of transfer. However, the sequences of gene transfer by all Hfrs proved to be circular permutations of a single sequence, establishing the circularity of the chromosome map. When an F' transfers chromosome, it does so from a fixed origin and with a transmission gradient like an Hfr, but the transfer frequency is intermediate between that of F^+ and an Hfr.

One further distinction between F^+, Hfr and F' mediated transfer of chromosome is that the products of such crosses involving F^+ or F' are generally fertile, while those from an Hfr cross are not.

Much work has been done on the genetics of F^+ transfer (Achtman

et al., 1972) and on the properties of F^+, F' and Hfr strains. Jacob and Wollman (1961) proposed that the fertility of F^+ cultures is entirely due to the formation of stable Hfr clones and it was suggested that F's integrated into the chromosome by reciprocal recombination to become donors (Pittard and Adelberg, 1963; Scaife and Gross, 1963). It was also found that in the formation of F's from an Hfr those extra genes carried on the F' were deleted from the chromosome and furthermore that such strains (primary F's) are no better at mobilising the chromosome than an F^+ strain (Pittard and Ramakrishnan, 1964).

A model suggested by Campbell (1961) postulated that the integration of episomes in general results from a single reciprocal recombination event between the circular episome DNA molecule and the circular bacterial chromosome. It became widely assumed that such a recombination event between transmissible plasmids and the chromosome was a prerequisite for chromosome transfer. Clowes and Moody (1966) showed that the chromosome mobilisation frequencies of F^+ and F' were both reduced to the same level in a *recA* strain. Later work (Wilkins, 1969) confirmed this and showed the *recB* and *recC* genes to be only slightly, if at all, involved.

Clearly, then, recombination via the *recA* pathway is important in the formation of chromosome donors. However, chromosome transfer by F and F' is not abolished in an *recA* strain, and the residual transfer is not thought to be due to leakiness of the *recA* mutation (Moody and Hayes, 1972). Also it was claimed that only 10 to 20% of the fertility of an F^+ culture can be accounted for by the formation of stable Hfr clones (Broda, 1967; Curtiss and Renshaw, 1965 and 1969). Thus it may be that there is, in addition to the stably integrated Hfr state, a more transient association between plasmid and chromosome (or possibly a reversible intermediate stage in Hfr formation) which is responsible at

least in part for chromosome mobilisation by F^+ and F' strains. Work by Evenchik *et al.* (1969) on the stimulation of chromosome transfer by low doses of ultraviolet light led them to postulate such an association. This was reiterated by Moody and Hayes (1972).

The *recA* pathway is not essential for mobilisation by F, or for Hfr formation, and therefore an "illigitimate" recombination event not requiring extensive DNA homology or a functional *recA* gene may be involved. [However, when an F' integrates into the chromosome of a *recA* mutant to form an Hfr, it does so at the site of plasmid-chromosome homology (DeVries and Maas, 1971)].

Evidence for such illegitimate recombinations came with the introduction of the heteroduplex technique (Davis and Davidson, 1968; Westmorland *et al.*, 1969). This allowed visualisation of a certain class of mutants first described by Lederberg (1960) and led to the discovery of genetic elements known as insertion sequences or ISs. [ISs are discussed more fully in Chapter 4. For a review see Starlinger and Saedler, 1976]. ISs are capable of illegitimate, *recA* independent recombinations (Reif and Saedler, 1975; Starlinger and Saedler, 1976) and so far five classes have been designated (IS1-IS5 Szybalski, 1977). Multiple copies of IS1 and IS2 have been detected in the *E. coli* chromosome (8 and 5 approximately, Saedler and Heiss, 1973). Heteroduplex analysis of F's (Hu *et al.*, 1975a and b) showed them to contain copies of IS1 and IS2 and that IS2 was involved in Hfr formation, at least in some cases. This followed earlier genetic (Broda *et al.*, 1972) and molecular (Sharp *et al.*, 1972) work demonstrating that there were different sites on F at which it could integrate into the chromosome. Subsequent studies have confirmed the involvement of insertion sequences on F in Hfr and F' formation, (see Ohtsubo and Ohtsubo, 1977) although the mechanisms are only beginning

to be understood.

Because the F factor has a limited host range (Datta and Hedges, 1972) it could not be used to elucidate the genetic map of other bacterial genera as it had been in *E. coli*. The search for suitable alternatives among R plasmids was often unsuccessful. With the advent of restriction enzyme technology, however, the possibility arose of engineering such plasmids *in vitro*. Plasmid RP4 (IncP) seemed particularly suitable for this because of its extraordinarily wide host range (Datta *et al.*, 1971; Datta and Hedges, 1972; Olsen and Shipley, 1973) and because, in spite of its size (36 Md.), it has only a single site for the restriction enzyme *Eco*R1 (Jacob and Grinter, 1975). However, R' plasmids made from RP4 and either *Proteus mirabilis* or *Rhizobium leguminosarum* chromosomal DNA, failed to mobilise the chromosome in those organisms above the very low frequency of mobilisation brought about by RP4 itself (Jacob *et al.*, 1976). The authors inferred that mobilisation needed both insertion sequences and DNA homology, and the probable lack of ISs associated with the inserted DNA on the hybrid plasmids accounted for their failure to act as sex factors. This was supported when R68.44 and R68.45 (two derivatives of R68 - a P-group plasmid very similar to RP4 - which have enhanced chromosome donor abilities in *Pseudomonas aeruginosa*) were shown to differ from the parent plasmid consistently with their having acquired a DNA fragment similar in size to an insertion sequence (Jacob *et al.*, 1977).

The insertion of *E. coli* chromosomal DNA into RP4 (Barth, 1979) produced plasmids which were good mobilisers of the *E. coli* chromosome and behaved analogously to F's. Since the hybrids contained only short chromosomal sequences (2.3 to 8.5 Md.), it seemed improbable they had all acquired insertion sequences. Thus it seems that the requirements for chromosome mobilisation differ between bacterial

Table 5.1. Chromosome mobilisation in various bacterial genera

Plasmid	Strain	Nature of chromosomal transfer	Size of transferred chromosomal DNA	Fate of plasmid after mobilisation	References
RP4	*P. aeruginosa*	Almost undetectable	-	-	Stanisich and Holloway (1971)
RP4	*R. leguminosarum*	Undetectable	-	-	Beringer (1974)
RP4	*R. meliloti*	Random (multiple origins)	Large	Not tested	Meade and Signer (1977)
RP4	*A. calcoaceticus*	Polarised. At least 2 origins	Probably large	Frequently wholly lost	Towner and Vivian (1976a and b, 1977)
RP4	*E. coli*	Almost undetectable	Not tested	Not tested	Watson and Scaife (1977); Boucher *et al.* (1977)
RP4	*P. mirabilis*	Almost undetectable	-	-	Jacob *et al.* (1976)
RP4-PM1[1]	*P. mirabilis*	Almost undetectable	-	-	Jacob *et al.* (1976)
RP4-R11[2]	*R. leguminosarum*	Almost undetectable	-	-	Jacob *et al.* (1976)
R68.45[3]	*P. aeruginosa*	Random (multiple origins)	Small	Partly or wholly lost	Haas and Holloway (1976)
R68.45	*R. leguminosarum*	Random (multiple origins)	Up to 1/7 of chromosome	95% of recombinants R^+	Beringer and Holloway (1976); Beringer *et al.* (1978)
R68.45	*R. meliloti*	Random (multiple origins)	Large	Partly or wholly lost in 10-85% of recombinants	Meade and Signer (1977); Kondorosi *et al.* (1977)
R68.45	*E. coli*	Promotes chromosome transfer	Not tested	Not tested	Beringer and Hopwood (1976)
R68.45	*P. mirabilis*	Undetectable	-	-	Coetzee (1978a)
R772[4]	*P. mirabilis*	Random (multiple origins)	Small	Inherited unaltered	Coetzee (1978a)

Table 5.1 Continued. Chromosome mobilisation in various bacterial genera

Plasmid	Strain	Nature of chromosomal transfer	Size of transferred chromosomal DNA	Fate of plasmid after mobilisation	References
P-*lac*R1*drd*19[5]	*P. mirabilis*	Polarised	< 10'	Inherited unaltered	Coetzee (1978b)
R1	*E. coli*	Polarised	Large	Generally inherited unaltered	Pearce and Meynell (1968)
pTB21[6]	*E. coli*	Polarised	Large	Inherited unaltered	Barth (1979)
pTB51[6]	*E. coli*	Polarised	Large	Inherited unaltered	Barth (1979)

1 An R' generated *in vitro* consisting of RP4 and inserted *Proteus mirabilis* chromosomal DNA.

2 An R' generated *in vitro* consisting of RP4 and inserted *Rhizobium leguminosarum* chromosomal DNA.

3 A derivative of R68 [IncP, very similar to RP4 (M. Van Montagu, personal communication)] with enhanced chromosome mobilising ability in Pseudomonas possibly resulting from the acquisition of an insertion sequence (Jacob *et al.*, 1977).

4 IncP-1 in *Pseudomonas* (equivalent to IncP in *E. coli*). Molecular weight 40 Md. Confers kanamycin resistance.

5 Recombinant between P-*lac* and R1*drd*19 isolated by a transductional technique (Coetzee, 1974).

6 R's generated *in vitro* consisting of RP4 and inserted *E. coli* chromosomal DNA.

genera. Table 5.1 attempts to summarise findings on chromosomal mobilisation using a variety of plasmids in several genera. No clear pattern emerges and it suggests that, like conjugation itself, chromosome transfer is a widespread phenomenon but it may be highly adapted to the genus in which it is taking place.

In the experimental work described below, the problem of chromosome mobilisation was approached from two directions. Firstly, using *in vitro* genetic manipulations, a test system was constructed to allow the separate assessment of the importance in mobilisation of

i) extensive plasmid-chromosome homology

ii) the insertion sequence IS1

iii) the *recA* gene.

Secondly, an R' formed *in vivo* was characterised.

A. *In vitro* R's

RP4 was chosen for the *in vitro* studies for several reasons:

1) It has a single cut site for the restriction endonuclease *Hind*III which occurs within the gene coding for kanamycin resistance (Barth and Grinter, 1977). Hence "insertional inactivation" of the kanamycin resistance by any foreign DNA inserted at the *Hind*III site makes the detection of hybrid plasmids very simple.

2) It does not contain IS1 or IS2 (Starlinger and Saedler, 1976).

3) It has already been used to make R's (Jacob *et al.*, 1976; Barth, 1979) with conflicting results as to their ability to mobilise bacterial chromosomes.

4) The native plasmid mobilises the *E. coli* chromosome at a barely detectable frequency (see Table 5.1).

It would have been possible to get copies of insertion sequences into RP4 by *in vivo* techniques, but without control over the site or orientation of the insertion. A better method was to use a class of mutants

of bacteriophage λ first isolated by Brachet *et al.* (1970). These were shown to contain IS1(λr14) or IS2 (λr32) by Fiandt *et al.* (1972). As IS1 is not cut by *Hind*III (Szybalski, 1977), a *Hind*III digest of λr14 DNA will contain fragments with an intact copy of IS1 which can be ligated into a *Hind*III-generated linear RP4 molecule. A plasmid created in this way, apart from carrying an insertion sequence, has extensive homology with the chromosome of a strain lysogenic for λ, but none with a λ-free strain. Thus the importance of homology and IS1 to mobilisation can be tested both separately and together in *recA*$^+$ and *recA* strains.

B. *In vivo* R'

R68.44p*argF*[1] is a derivative of R68.44 (Haas and Holloway, 1976) made by crossing *Pseudomonas aeruginosa* strain PAC174(R68.44) with *E. coli* strain 4100, selecting for arginine independence. The recombinant plasmid obtained in this way was shown to have acquired a piece of the *Pseudomonas* chromosome of 92.4 Mdal. The isolation and characterisation of R68.44p*argF* is described by Hedges and Jacob, 1977, except it was then called R68.44p*argG*. It has been renamed since it was shown that *E. coli* strain 4100 is an *argF* and not *argG* mutant (Nicolas Glandsdorff, personal communication). It has been shown subsequently that R68.44p*argF* also carries the *Pseudomonas argG* genes (B. W. Holloway, personal communication; N. Glandsdorff, personal communication).

The experimental work consisted of using P1*kc* transduction to shorten the recombinant plasmid (Pittard and Adelberg, 1963). An analysis of the various deletions produced allowed the site of insertion of the *Pseudomonas argF* DNA into R68.44 approximately to be located.

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1 Throughout this chapter the nomenclature of the arginine genes is that used for *E. coli* (Bachman *et al.*, 1976).

Transductants which were still self-transmissible were tested for the retention of the *argG* function. Molecular and genetic studies were carried out on selected transductants.

Results

A. Experiments on *in vitro* generated RP4-λ hybrids

[In this section the eight fragments produced by *Hind*III digestion of linear λ DNA are numbered 1-8 in descending order of molecular weight, as shown in Figure 5.1.]

1. Formation of hybrids. The technique for restriction endonuclease cleavage of DNA and subsequent ligation to form biologically functional recombinant plasmid molecules was derived from Cohen *et al.* (1973) and is described in Chapter 1. RP4 is cut once by *Hind*III (Barth and Grinter, 1977) and bacteriophage λ DNA at seven sites (Robinson and Landy, 1977), while there are no cut sites for *Hind*III on IS1 (Szybalski, 1977). A genetic and restriction map of λ is shown in Figure 5.1. This also shows the site of insertion of IS1 in λ*r*14 which is in *Hind*III fragment 3 (molecular weight 4.8 Md or 4.3 Md without IS1).

Two methods were adopted to incorporate λ restriction fragments into RP4. Firstly, λ DNA was digested with *Hind*III and the fragments separated by electrophoresis when the required pieces could be eluted from the gel (Chapter 1) and ligated with *Hind*III cut RP4. The recovery of material from the gel (as estimated by adsorption at 260 nm) was good (60-80%) and the ligation done under conditions described by Dugaiczyk *et al.*, 1975, which should have meant that a quarter to one third of the covalently closed circular (CCC) plasmid molecule recovered after ligation were recombinants. After transformation with such ligated DNA, however, less than 1% of the ampicillin resistant colonies contained a hybrid plasmid [detected by sensitivity to kanamycin caused by insertional inactivation of the kanamycin resistance gene (Barth and Grinter, 1977)]. The reason for the low yield of recombinants is not known. Possibly the extraction of restricted DNA from agarose damages

Figure 5.1: Genetic and restriction map of bacteriophage λ.

The λ genome is the horizontal line which is marked with the location of some phage genes. Above it is the HindIII digestion pattern, and below it, that of EcoRI and BglII. The HindIII fragments are numbered 1-8 in descending order of molecular weight. Data are from Robinson and Landy, 1977 (HindIII sites); Thomas and Davis, 1975(EcoRI sites); Kamp et al., 1977 (BglII sites); Echols and Murialdo, 1978 (gene location); Gottesman and Adhya, 1977 (IS1 position in λrif4); K.Murray, personal communication (molecular weight of λ).

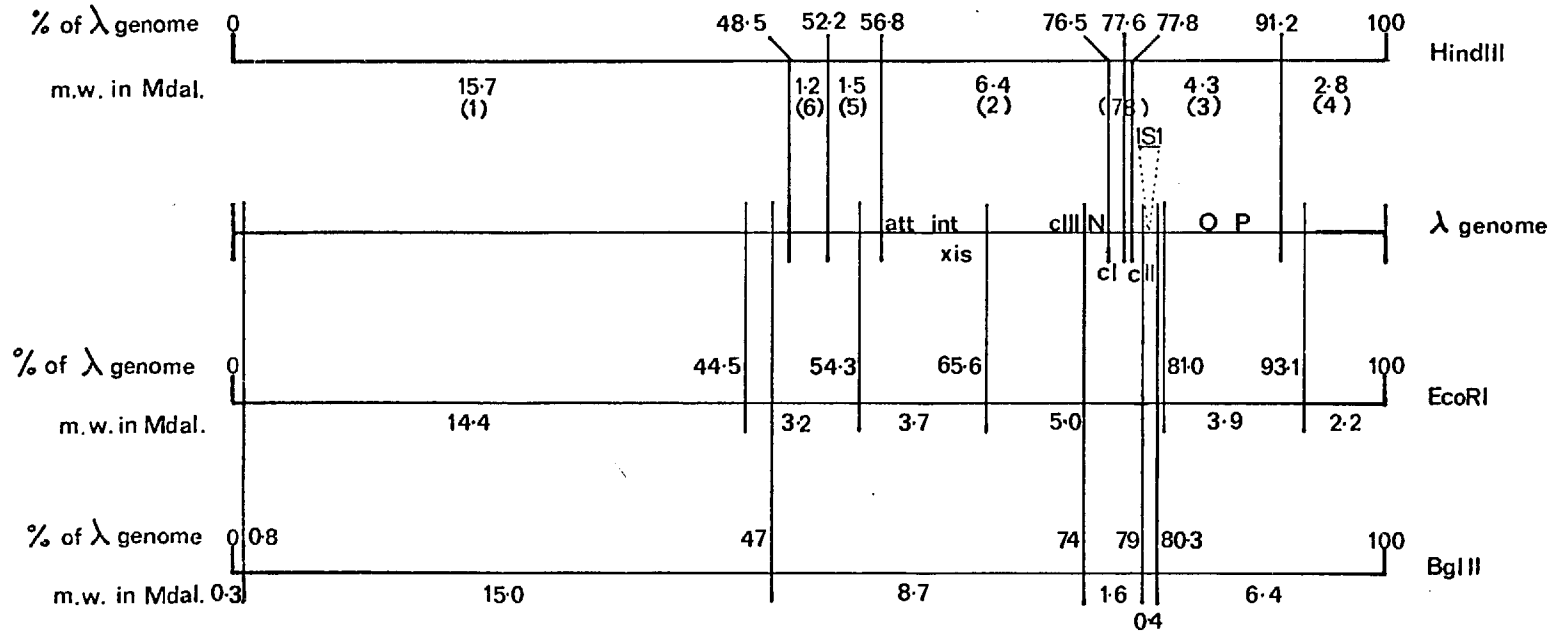


Figure 5.1 : Genetic and restriction map of bacteriophage λ .

the *Hind*III generated sticky ends; other work has shown this to be unlikely (P. Bergquist, personal communication). Another possibility is that the recombinants are unstable, or largely non-viable, or somehow selected against by the transformation procedure. None of these seem likely because of the success in isolating the recombinants by another approach (see below). The most probable explanation is that the source of ultra-violet radiation used to visualise the λ fragments after gel electrophoresis was too powerful and rendered the DNA biologically inactive. This possibility was not tested. This technique was used to make only the three RP4-λ hybrids carrying the *Hind*III fragment 3 of λ without IS1 (pHH6000-pHH6002, Table 5.2).

The remaining RP4-λ hybrids were made by mixing RP4 DNA with λ*r*14 DNA, cutting with *Hind*III and ligating. Concentrations were adjusted to give a j:i ratio of 2 (Dugaiczyk *et al.*, 1975) which meant that about 30% of the RP4 CCC molecules recovered after ligation should have contained inserted λDNA. In fact after transformation with this material about 10% of the ampicillin resistant colonies were kanamycin sensitive indicating that they carried inserted DNA.

About 40 kanamycin sensitive clones isolated in this way were subjected to electrophoresis using the single colony lysis technique (Chapter 1). Ten were selected which had plasmids of molecular weight between 36 and 45 Mdal (data not presented) and plasmid DNA was prepared from them by caesium chloride-ethidium bromide density gradient centrifugation. Purified plasmid DNA was cut with *Hind*III and run on a horizontal gel against *Hind*III cut λ*r*14 DNA as a standard. One plasmid was found containing the IS1-carrying fragment (pHH6003, Table 5.2). Several plasmids contained other λ fragments, including pHH6015 which contains fragment 2 carrying the λ attachment site (Figure 5.1), and some contained two inserted pieces. One of this latter category

Table 5.2. Plasmids constructed *in vitro*

Plasmid	Method of construction[1]	*Hind*III fragment(s) carried[2]	Orientation[3]
pHH6000	a	3(without IS1)	A
pHH6001	a	3(without IS1)	B
pHH6002	a	3(without IS1)	B
pHH6003	b	3(+ IS1)	A
pHH6004	c	3(+ IS1)	A
pHH6005	c	3(+ IS1)	A
pHH6006	c	3(+ IS1)	B
pHH6007	c	3(+ IS1)	A
pHH6008	c	3(+ IS1)	A
pHH6009	c	3(+ IS1)	B
pHH6010	c	3(+ IS1)	A
pHH6011	c	3(+ IS1)	B
pHH6015	b	2	-
pHH6020	b	3(+ IS1) + 6	-

1 The methods used to construct the various plasmids were as follows:

a Ligation of *Hind*III cut RP4 with λ *Hind*III fragment 3 (see footnote 2) extracted from an agarose gel as described in Chapter 1.

b Ligation of *Hind*III cut RP4 with *Hind*III cut λ DNA.

c *Hind*III digestion and re-ligation of pHH6020.

2 The eight λ fragments given by *Hind*III digestion are numbered 1-8 in descending order of molecular weight (see Figure 5.1).

3 The meaning of orientation A and B is explained in the text and Figure 5.2. - means orientation not determined.

(pHH6020) had fragment 3 (including IS1) and fragment 6.

DNA from pHH6020 was *Hind*III digested, ligated and used for transformation. 9% (10/116) of the ampicillin resistant transformants were kanamycin sensitive. Eight of the ten were found to contain only the inserted IS1-carrying λ fragment (pHH6004-pHH6011, Table 5.2).

2. Orientation of the inserted λ DNA. The IS1-carrying *Hind*III fragment of λrl4 has an *Eco*R1 cut site near one end (Figure 5.1). IS1 is not itself cut by *Eco*R1 (Szybalski, 1977) and the single *Eco*R1 site on RP4 is situated 12.9 Md. away from the *Hind*III site (Barth and Grinter, 1977). Therefore, cutting the RP4-λ hybrids with *Eco*R1 will yield two fragments whose relative sizes depend on the orientation of the λ *Hind*III fragment 3 inserts (Figure 5.2). This also serves to check that there is only a single copy of the λ fragment per plasmid, as any extra copies would yield additional gel bands after *Eco*R1 digestion. The results are summarised in Table 5.2.

3. Chromosome mobilisation with the RP4-λ hybrids. Seven plasmids carrying λ *Hind*III fragment 3 were selected. These included the three (pHH6000-6002) which have inserted λDNA only (no IS1) and four others (pHH6003, 6004, 6009 and 6011) which in addition carry IS1. pHH6000, 6003 and 6004 have the inserted material in orientation A, and the rest have it in orientation B (Figure 5.2). Also used was pHH6015 which carries the λ attachment region on λ *Hind*III fragment 2. *Hind*III and *Eco*R1 digests of these plasmids after gel electrophoresis are shown in Plates 5.1 and 5.2.

The plasmids were transferred by plate matings into W3110T$^-$, W3110T$^-$(λ), W3110*recA* or W3110*recA*(λ) using tetracycline selection. Transconjugants were purified on selective medium and tested for antibiotic resistance pattern, nutritional requirements, λ immunity/sensitivity and ultraviolet sensitivity/resistance.

Figure 5.2: Orientation of the λ *Hind*III fragment 3 inserted into RP4

The outer circle in both diagrams represents the plasmid DNA molecule with the inserted λ DNA shown as thickly-drawn regions between the two *Hind*III cut sites. Molecular weights of the various sections are shown in Megadaltons (Md) inside the inner circle. *Eco*R1 cleaves the 4.8 Md λ insert into 1.5 and 3.3 Md pieces, with IS1 carried on the 1.5 Md piece. RP4 itself is cut once by *Eco*R1, 12.9 Md from the *Hind*III site. *Eco*R1 digestion of the plasmids yields the following fragments:

orientation A - 26.4 and 14.4 Md.

orientation B - 24.6 and 16.2 Md.

If IS1 (0.5 Md) is not on the inserted λ DNA, the fragments after *Eco*R1 digestion are:

orientation A - 26.4 and 13.9 Md.

orientation B - 24.1 and 16.2 Md.

Hence the two orientations are distinguishable by agarose gel electrophoresis of the fragments of plasmid DNA generated by *Eco*R1 digestion. Data are from Robinson and Landy, 1977 (*Hind*III sites on λ DNA); Barth and Grinter, 1977 (molecular weight of RP4 and position of *Hind*III and *Eco*R1 sites) and Fiandt *et al.*, 1972 (molecular weight of IS1 and site of insertion in λr14).

Figure 5.2 : orientation of the λ HindIII fragment 3 inserted into RP4 .

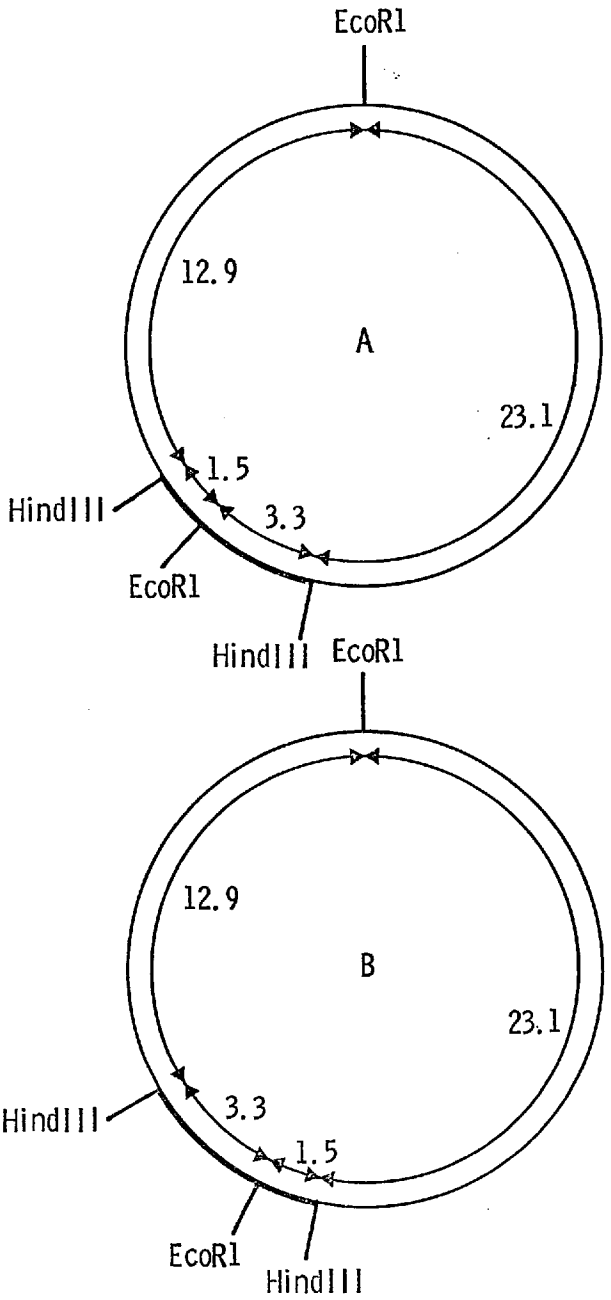


Plate 5.1. Electrophoresis of *HindIII* cut plasmid DNA

The DNAs were prepared, digested and run as described in Materials and Methods (Chapter 1). The gel was 0.8% agarose and electrophoresis was at 150V for 4 hours. Contents of the tracks are as follows: 1. pHH6015; 2. λ; 3. pHH6000; 4. pHH6001; 5. pHH6002; 6. λ*r*14; 7. pHH6003; 8. pHH6004; 9. pHH6009; 10. pHH6011; 11. RP4.

RP4 gives a single fragment after *HindIII* cutting, while all the other plasmid DNAs are resolved into two fragments, the larger of which has the same mobility as RP4. pHH6000-pHH6002 (tracks 3-5) contain the λ*Hind*III fragment 3 while pHH6003, 6004, 6009 and 6011 (tracks 7-10) contain λ*r*14 *Hind*III fragment 3 which is larger because it carries IS1. pHH6015 (track 1) has the λ *Hind*III fragment 2. (λ DNA in track 2 has not been completely digested, resulting in the faint band seen running between the first and second strong bands. Complete digestion gives a series of bands whose intensity decreases with increasing mobility, as in track 6).

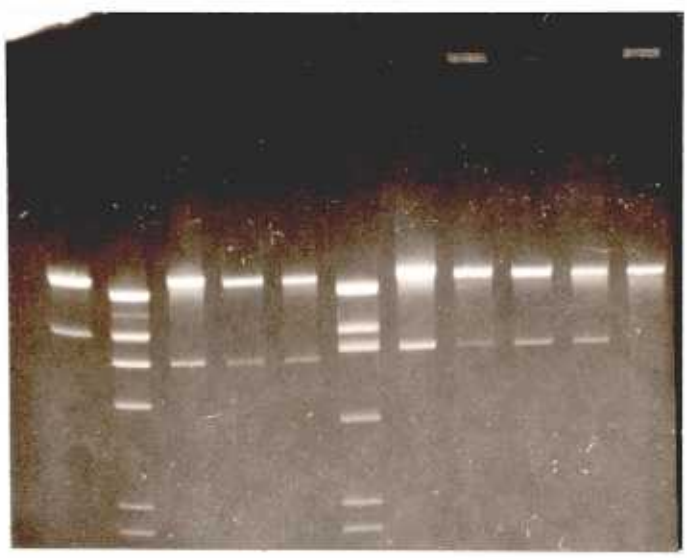
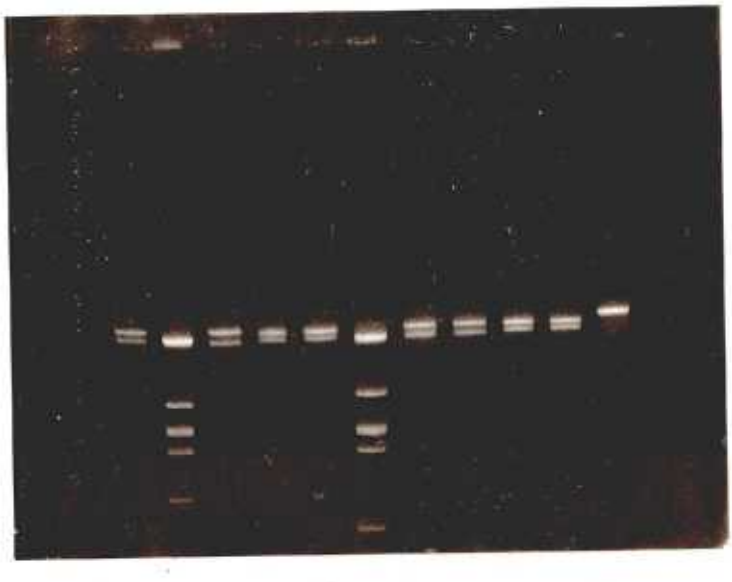


Plate 5.2. Electrophoresis of *Eco*R1 cut plasmid DNA

The DNAs were prepared, digested and run as described in Materials and Methods (Chapter 1). The gel was 0.7% agarose and electrophoresis was at 150V for 6 hours. Contents of the tracks are as follows: 1. pHH6015; 2. λ; 3. pHH6000; 4. pHH6001; 5. pHH6002; 6. λ*r*14; 7. pHH6003; 8. pHH6004; 9. pHH6009; 10. pHH6011; 11. RP4.

RP4 gives a single fragment after *Eco*R1 cutting, while all the other plasmid DNAs are resolved into two fragments. The separation between the fragments depends on the orientation of the inserted λ DNA (Figure 5.2). pHH6000, pHH6003 and pHH6004 (tracks 3, 7 and 8) give more widely separated bands (indicating orientation A) while pHH6001, 6002, 6009 and 6011 (tracks 4, 5, 9 and 10) give bands running closer together (indicating orientation B). IS1 is present on λ*r*14 *Eco*R1 fragment 2, hence it runs more slowly than the corresponding λ fragment. pHH6015 (track 1) contains λ *Hind*III fragment 2 (see Plate 5.1) which carries an *Eco*R1 site which is located centrally upon it (Figure 5.1). Consequently, *Eco*R1 digestion of the plasmid DNA gives two fragments but says nothing about the orientation of the insert.



The recipient for chromosomal mobilisation in most cases was J62-2. This was chosen because it has two markers (*proC* and *trp*) equidistant (about 10') from and on either side of the *gal-bio* region where λ normally inserts in the *Escherichia coli* chromosome (Bachman, 1972 and Bachman *et al.*, 1976). The multiply marked AB1157 and its *recA* derivative AB2463 were also used as recipients in some cases. Crosses were performed in broth (as described in Chapter 1) for 3 hours. Viable counts were made on the donors, and the frequency of both plasmid and chromosome transfer was estimated after mating. Chromosome transfer is expressed as a fraction of plasmid transfer, thereby eliminating variation due to differences in plasmid transfer frequency. Controls were plated to estimate the reversion frequency of the recipient and this was allowed for in the calculations. The results are shown in Tables 5.3 and 5.4. Where J62-2 was the recipient, the detection of *pro*$^+$ recombinants was less sensitive than that of *trp*$^+$ because of the higher reversion frequency (2.5×10^{-7} as against 5×10^{-9}).

Apart from those with pHH6015 which are discussed later, the results in Table 5.3 show:

1. There was no detectable mobilisation by RP4 alone.
2. There was no detectable mobilisation from *recA* donors.
3. There was no detectable mobilisation from *recA*$^+$ λ$^-$ donors by plasmids without IS1.
4. All plasmids carrying inserted λ DNA mobilised the chromosome from *recA*$^+$ λ lysogens, the direction of mobilisation depending on the orientation of the insert. [Plasmids with the insert in orientation A (Figure 5.2) transferred *pro*$^+$ as a proximal marker, while those in orientation B transferred *trp*$^+$ proximally].

5. Plasmids carrying IS1 mobilised the chromosome from *recA*$^+$ λ^- strains at a frequency (for *trp*$^+$ transfer) roughly 1% of that seen from *recA*$^+$ λ lysogens (compare pHH6009 and 6011 in W3110T$^-$ with the same plasmids in W3110T$^-$(λ)).

6. The high level of mobilisation promoted by plasmid-chromosome homology was not enhanced by the additional presence of IS1 on the plasmid.

Thus, mobilisation from *recA*$^+$ donors correlates with the presence of plasmid-borne IS1 and/or more extensive plasmid-chromosome homology. Interaction between longer homologous sequences on the plasmid and chromosome promotes chromosome transfer at least 100 times more efficiently than IS1 alone, and there is no synergistic effect from having homology and IS1 together on the plasmid. The orientation of inserted λ DNA determines the direction of transfer.

If the system is analogous to F'-mediated chromosome transfer, then the failure to show any mobilisation from *recA* donors may be only a reflection of the lower transfer frequency of RP4 (1×10^{-3} compared with $1\text{-}10 \times 10^{-1}$ for F) and the consequent increase in the lowest levels of mobilisation detectable. Mobilisation by F is thought to be due to interaction between insertion sequences on the plasmid and the bacterial chromosome (Ohtsubo and Ohtsubo, 1977) and is substantially reduced in a *recA* background (Moody and Hayes, 1972). A proportionate reduction in the system under study here would put the number of recombinants below the level of detection. Taking the analogy further, this also means that the low levels of *trp* transfer by plasmids with λ + IS1 inserts in orientation A are IS1 mediated rather than their being transferred as terminal markers, because there is a 10^4 drop in transfer frequency between proximal and terminal marker transfer mediated by F'. A similar drop in this system would mean that the frequency of terminal

Table 5.3. Chromosome transfer by RP4 and RP4-λ hybrids to J62-2

Donor	Plasmid-borne *Hind*III fragment of λ and [orientation]	Chromosome transfer (per plasmid transfer x 10^5)	
		pro$^+$ transfer	*trp*$^+$ transfer
(RP4)W3110T$^-$	-	undetectable[1]	undetectable[2]
(pHH6000)W3110T$^-$	3[A]	undetectable	undetectable
(pHH6001)W3110T$^-$	3[B]	undetectable	undetectable
(pHH6002)W3110T$^-$	3[B]	undetectable	undetectable
(pHH6003)W3110T$^-$	3(+ IS1)[A]	undetectable	12
(pHH6004)W3110T$^-$	3(+ IS1)[A]	undetectable	11
(pHH6009)W3110T$^-$	3(+ IS1)[B]	undetectable	4
(pHH6011)W3110T$^-$	3(+ IS1)[B]	undetectable	8
(RP4)W3110T$^-$(λ)	-	undetectable	undetectable
(pHH6000)W3110T$^-$(λ)	3[A]	430	undetectable
(pHH6001)W3110T$^-$(λ)	3[B]	undetectable	360
(pHH6002)W3110T$^-$(λ)	3[B]	undetectable	620
(pHH6003)W3110T$^-$(λ)	3(+ IS1)[A]	260	2
(pHH6004)W3110T$^-$(λ)	3(+ IS1)[A]	580	6

Table 5.3 Continued. Chromosome transfer by RP4 and RP4-λ hybrids to J62-2

Donor	Plasmid-borne *Hind*III fragment of λ and [orientation]	Chromosome transfer (per plasmid transfer x 10^5)	
		pro$^+$ transfer	*trp*$^+$ transfer
(pHH6009)W3110T$^-$(λ)	3(+ IS1)[B]	undetectable	1,100
(pHH6011)W3110T$^-$(λ)	3(+ IS1)[B]	undetectable	490
(pHH6015)W3110T$^-$(λ)	2	920	8
(RP4)W*recA*	-	undetectable	undetectable
(pHH6000)W*recA*	3[A]	undetectable	undetectable
(pHH6003)W*recA*	3(+ IS1)[A]	undetectable	undetectable
(RP4)W*recA*(λ)	-	undetectable	undetectable
(pHH6000)W*recA*(λ)	3[A]	undetectable	undetectable
(pHH6003)W*recA*(λ)	3(+ IS1)[A]	undetectable	undetectable
(pHH6015)W*recA*(λ)	2	230	undetectable

1 Less than 10.

2 Less than 0.2.

marker transfer would be well below the reversion frequency of the recipient.

That marker transfer is orientated was supported by looking at the co-transfer of unselected markers to the *pro*$^+$ and *trp*$^+$ recombinants described in Table 5.3. 20/30 colonies that had received *pro*$^+$ from (pHH6003)W3110T$^-$(λ) were now also *lac*$^+$, while 30/30 *trp*$^+$ colonies from (pHH6009)W3110T$^-$(λ) were *lac*$^-$. Consideration of the relative positions of these markers on the *E. coli* genetic map (Bachman *et al.*, 1976) suggests that marker transfer is in an anticlockwise direction from pHH6003 and probably clockwise from pHH6009.

Turning to pHH6015 (carrying the λ attachment region), chromosome mobilisation is almost unaffected by the *recA* locus. Proline independence is transferred proximally from both *recA*$^+$ and *recA* donors with only a fourfold drop in frequency in the *recA* strain. The level of transfer seen from the *recA*$^+$ donor is due to the effects both of extensive plasmid-chromosome homology and interactions between the λ attachment sites on the plasmid and chromosome. In the *recA* donor, only the latter mechanism is active. The frequency of *pro*$^+$ transfer is comparable to that determined by pHH6000, 6003 and 6004. Consequently the same arguments as used above regarding terminal marker transfer can be applied. The low frequency of *trp*$^+$ transfer by pHH6015 in W3110T$^-$(λ) is presumably due to the λ attachment site on the plasmid interacting with the chromosome somewhere other than the *gal-bio* region which carries the site for normal λ attachment. Abnormal insertions by λ at all loci account for between 0.1 and 1% of all λ insertions (Enquist and Weisberg, 1977). A site for such an insertion suitably placed near the tryptophan region would account for *trp*$^+$ transfer by pHH6015. Failure of this plasmid to transfer *trp*$^+$ from W3110*recA*(λ) is not understood unless the abnormal inter-

actions between λ and the chromosome in some way depend on *recA*.

[A further property of pHH6015 is that it appears to confer λ immunity on those cells carrying it. All attempts by both plate and broth matings to transfer it to W3110T⁻ or W3110*recA* gave transconjugants, normal in all other respects, which were λ^{imm}. Of the genes involved in λ lysogeny and superinfection inhibition, only C_{III} is on the λ fragment carried by pHH6015 (Figure 5.1). If the C_{III} function is expressed by strains carrying this plasmid, this may be enough to tip the balance towards lysogeny rather than lysis when such strains are infected by λ phage (Echols and Murialdo, 1978). If this were true, elimination of the plasmid should restore λ sensitivity. Accordingly, RP4 was introduced into pHH6015 containing strains, selecting for kanamycin resistance. This should eliminate pHH6015 by incompatibility, but 8/8 transconjugants tested retained λ immunity. It is possible that pHH6015 can integrate into the chromosome like λ. (Watson and Scaife, 1978, reported the chromosomal integration of another RP4 carrying the λ attachment site.) Replication of the integrated plasmid would lead to excessive and uncontrolled replication of the surrounding chromosome and the insertion would be unstable. Excision of the plasmid could leave the λ C_{III} region behind in the chromosome, resulting in λ immunity not being eliminated with the plasmid. This could be tested by P1 transduction of the λ attachment (*gal-bio*) region of the immune strain, looking for i) co-transduction of unselected λ immunity and ii) a decrease in co-transduction frequency between markers on either side of the chromosomal λ attachment site resulting from the presence of inserted DNA. This has not been tested.

Whatever the mechanisms involved, the experiments to show chromosome mobilisation by pHH6015 were done from strains which were genuine λ lysogens.]

4. Mechanism of chromosome mobilisation. The fate of the plasmid in the recipient after mobilisation, the polarity or randomness of marker transfer and the size and state of the transferred DNA all give indications as to the mechanism involved.

If transfer is by the R' integrating into the chromosome in a manner analogous to F's forming Hfrs, then chromosome recipients are unlikely to receive a complete plasmid genome. This seems to be the case in some of the examples in Table 5.1 where the plasmid is partially or wholly missing from the recipient. [In F' mediated chromosomal transfer, the recipient is normally fertile (i.e. F^+), but this is generally attributed to the high transfer frequency of F causing the chromosomal recipient to be infected with an autonomous F by a separate conjugation event. RP4 with its much lower transfer frequency should not show this property.] There is the possibility of autonomous and integrated plasmids co-existing in the same cell as suggested by Watson and Scaife (1978). Their results, however, could show that the integrated plasmid reversibly reforms an R' by excision at a high frequency.

If chromosome transfer is by interaction between homologous sequences on the plasmid and chromosome, then transfer should proceed from a fixed origin with a gradient of marker transfer frequency, the chances of a given marker being transferred varying inversely with its distance from the origin. Chromosomal transfer from multiple origins would not give such a gradient.

Finally, if chromosome transfer is by "pickup" by the plasmid of material from the chromosome as described by Olsen and Gonzalez, 1974, then only short sequences of chromosome would be transferred and the transfer would take place to *recA*$^+$ and *recA* recipients with equal frequency.

From the matings of (pHH6003) and (pHH6009) in W3110T$^-$(λ), Table 5.3, 30 *pro*$^+$ and 30 *trp*$^+$ J62-2 colonies were tested for antibiotic resistance. All 60 were resistant to ampicillin and tetracycline, and sensitive to kanamycin. 4/4 of the *trp*$^+$ colonies were found to transfer tetracycline resistance to C600 at a frequency of c.1 x 10^{-3} per donor per hour (typical of RP4) and all the transconjugants tested had the unselected ampicillin resistance. This confirms the existence of an autonomous plasmid in the conjugation recipients. R751(IncP) was transferred into the same four *trp*$^+$ colonies, selecting for trimethoprim resistance. Four colonies from each cross were purified and tested for antibiotic resistance. All 16 had lost ampicillin and tetracycline resistance while retaining tryptophan independence. Thus there was no evidence for an integrated plasmid which could co-exist with another autonomous P plasmid. Also, retention of tryptophan independence means the relevant genes were not on the plasmid but presumably part of the chromosome.

Table 5.4 shows the results of attempts to mobilise the chromosome into the multiply marked strain AB1157 and its *recA* derivative AB2463. As before, RP4 gave no detectable chromosome transfer. pHH6003, however, transferred the *proA*, *thr* and *argE* markers to AB1157 with a gradient of transmission frequencies indicating that there was a fixed origin of transfer situated between *pro* and *trp* and that the transfer of markers proceeded in an anticlockwise direction around the chromosome, transferring *thr* next and then *arg*. This supports conclusions drawn in section 3 about the orientation of chromosome transfer from strains carrying pHH6003 and further suggests that large segments of chromosome are transferred.

There was no transfer of chromosomal markers from (pHH6003) W3110T$^-$(λ) to AB2463 even though the plasmid itself was transferred

Table 5.4. Mobilisations into AB1157 and AB2463 (AB1157*recA*)

Donor	Recipient	Transfer frequenicies/plasmid transfer x 10^5		
		pro+ (6')[1]	*thr*+ (1')	*arg*+ (88')
(RP4)W3110T$^-$(λ)	AB1157	< 0.5	< 0.5	< 0.5
(pHH6003)W3110T$^-$	AB1157	7	7	< 0.5
(pHH6003)W3110T$^-$(λ)	AB1157	100	34	6
(pHH6003)W3110T$^-$(λ)	AB2463	< 0.5	< 0.5	< 0.5

1 Figures in brackets after the name of the transferred marker are the location in minutes on the *E. coli* genetic map. Data from Bachman (1972) and Bachman *et al.* (1976).

quite readily. Therefore, "pickup" of chromosomal markers by the plasmid is not the mechanism of chromosome transfer.

Summary of mobilisation results

Hybrids between plasmid RP4 and λrif4 *Hind*III fragment 3 (carrying IS1) can mobilise the chromosome of *recA*$^+$ strains lysogenic for λ. They behave like F's in that they give polarised transfer of large segments of chromosome from a fixed origin. The origin is at or near the chromosomal λ attachment site. The direction of marker transfer is determined by the orientation of the inserted λ DNA in the plasmid. In λ$^-$ strains with no extensive plasmid-chromosome homology there is a residual low level of chromosome transfer presumably mediated by IS1. Chromosomal recipients also inherit an autonomous plasmid, there being no evidence for an Hfr-like state. The association between plasmid and chromosome which mediates chromosome transfer involves DNA homology (because the origin is at or very near the site of homology) and some *recA* function, although recombination between plasmid and chromosome has not been proved. The association is perhaps a transient one like that described by Evenchik *et al.* (1969) and Moody and Hayes (1972).

Mobilisation is undetectable from *recA* donors but probably because of the low transfer frequency of RP4 compared to that of F. pHH6015, however, a plasmid carrying the λ attachment region, could mobilise the chromosome of a *recA* strain at a frequency comparable to the other hybrid plasmids in *recA*$^+$ λ lysogens.

Discussion

There are many similarities between RP4-λ mediated chromosome transfer and that of F's. Plasmid-chromosome homology and insertion sequences are involved both separately and together, with homology making the major contribution. The *recA* mutation causes a marked drop in mobilisation in both systems and both systems give polarised transfer

of chromosome, the direction depending on the orientation of the inserted homologous DNA.

Mobilisation by Hfr proceeds from a fixed point on the integrated F, suggesting there is a unique origin of transfer. Such an origin may exist even on non-self-transmissible plasmids (Warren and Sherratt, 1977) and is thought to represent a specific site on the plasmid where a single stranded nick is generated immediately prior to transfer of a single strand of DNA to the recipient. Synthesis of complementary strands then takes place in both donor and recipient (Vapnek *et al.*, 1971).

At least some F' mediated chromosomal mobilisation is by Hfr formation (Curtis and Renshaw, 1969), but no evidence was obtained in this study for RP4-λ molecules forming Hfrs. It is known, however, that RP4 can insert in the chromosome in some circumstances (Martin *et al.*, 1975; Watson and Scaife, 1978). When an Hfr transfers the chromosome, the recipient receives a complete integrated F factor only if the entire chromosome is transferred. Consequently, recombinants are rarely fertile. In contrast, F' mediated recombinants are generally capable of acting as donors themselves, but this is thought to be because they receive an autonomous F' as a separate event. Clearly when RP4-λ transfers chromosome, the much lower transfer frequency of the autonomous plasmid (10^{-3} instead of 10^{-1}-10^{0} for F) would make a secondary infection of a recombinant most unlikely.[1]

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[1] RP4 is present in several copies per cell and so a bacterium transferring chromosome by a newly-integrated plasmid could also transfer an autonomous plasmid. Consideration of pilus production by RP4 suggests this is improbable. At the very least, 14% of RP4-bearing cells are piliated (Barth *et al.*, 1978) and presumably capable of forming conjugation bridges with other cells, and yet transfer of the plasmid is seen from only one donor per thousand per hour. This cannot be because the conjugative junction is fragile, as this would make very unlikely the transfer of the large lengths of chromosome seen by Barth, 1979, and in this thesis. More probably, the generation of an origin of transfer on RP4 is a rare event and the probability of it happening more than once during a particular cellular union is low.

Table 5.1 shows instances of chromosome transfer by RP4 or closely related plasmids where the recipient rarely inherits the plasmid, or receives only a part of it. Such transfers are very likely due to Hfr formation in the donor and are clearly different from crosses involving RP4-λ plasmids where all the chromosomal recipients acquired a complete autonomous plasmid.

As an alternative to the above, the position of the transfer origin on RP4 may explain the observations. Considering the orientation of bacteriophage λ in the chromosome of a lysogen (Gottesman and Adhya, 1977) and the orientation of λ DNA in the RP4-λ plasmids, the structure of an Hfr formed by reciprocal recombination between the homologous sequences can be drawn (Figure 5.3). If the transfer origin is located as shown, then almost the whole of RP4 is transferred proximally in conjugation. The missing piece is part of a larger segment deleted from RP4δ1, a spontaneous mutant of RP4 (Barth and Grinter, 1978), and so is known to be non-essential. In this system all chromosome recipients also acquire the material for a functional RP4 molecule. (In other genera with RP4 or its close relatives, Table 5.1, integration occurs at other sites on the plasmid and hence only part of the plasmid is transferred proximally.) The weakness of this argument is that it is hard to see why the transferred linear RP4 should so readily recircularise since, after transfer, it no longer has homologous sequences at either end. The model predicts the plasmids found after recircularisation in chromosomal recipients would no longer have an intact transfer origin and should therefore be non-transmissible. Where tested (Results, Section 4) this was found not to be the case.

The model of Evenchik *et al.*, 1969, to explain chromosomal transfer by F' without Hfr formation, describes an exchange between homologous single strands on the plasmid and chromosome, but it predicts the con-

Figure 5.3: Structure of hypothetical RP4-λ Hfrs

Recombination between homologous sequences on plasmid and chromosome gives rise to chromosomally inserted plasmid molecules. E is the *Eco*R1 cut site on RP4, Ap, Tc and Km mark the sites of the ampicillin, tetraycline and kanamycin resistance genes, the latter having been split by the inserted λ DNA. The arrow-head shows the possible site and direction of the transfer origin. Data from Gottesman and Adhya, 1977 (orientation of λ in the chromosome); Barth and Grinter, 1977 (map of RP4) and Echols and Murialdo, 1978 (map of λ).

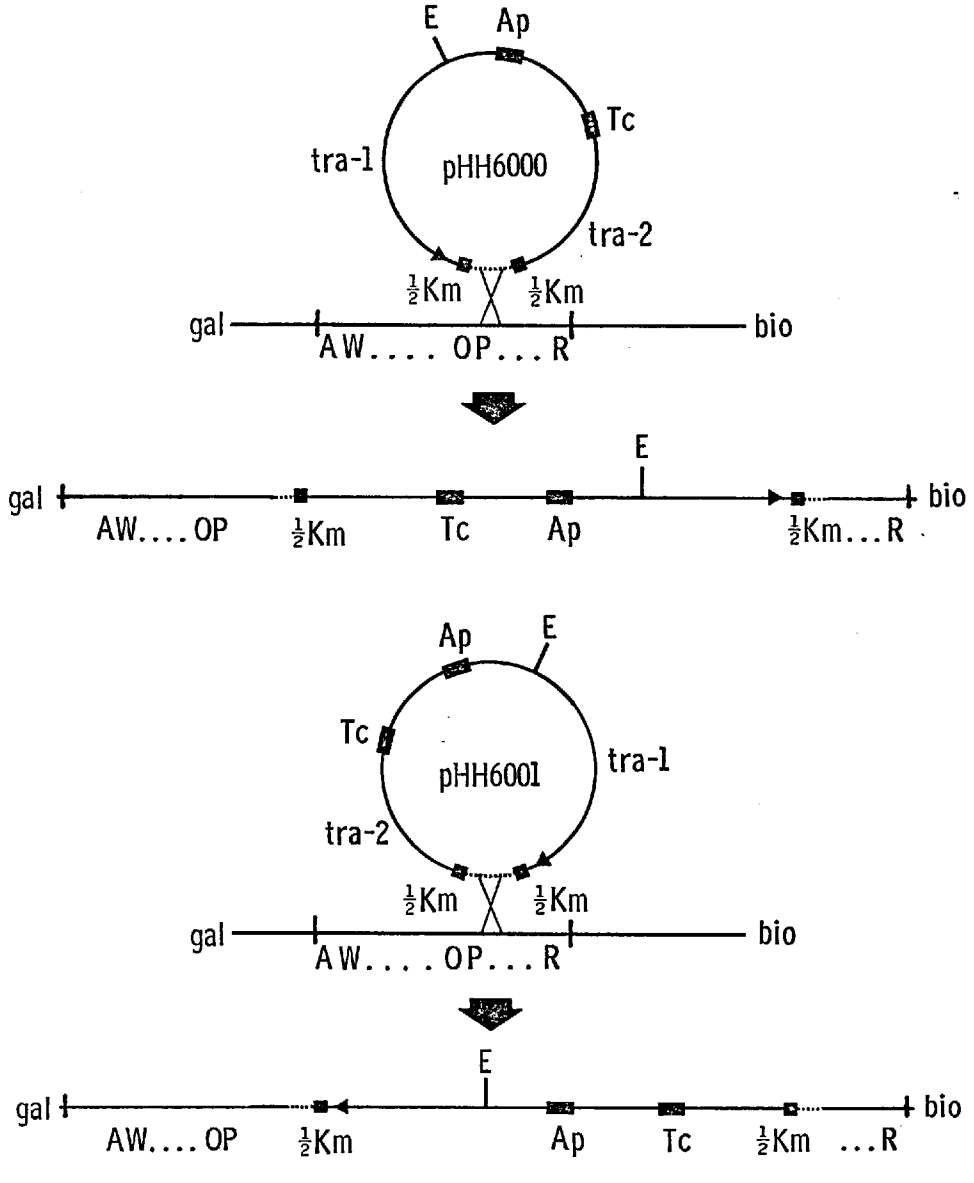


Figure 5.3 : Structure of hypothetical RP4-λ Hfrs .

jugation recipient will inherit only part of the plasmid, and so is not applicable to these results.

In addition to plasmid specified functions, the host strain must influence the nature and extent of chromosome transfer. RP4's generated by Jacob *et al.* (1976) in *Rhizobium* and *Proteus* spp. did not mobilise the chromosomes and the authors suggested this was because of the absence of insertion sequences on the cloned *Rhizobium* or *Proteus* DNA. Work presented here shows that in *E. coli* plasmid-borne DNA homology with the chromosome is sufficient by itself to promote mobilisation. In *Rhizobium* at least, failure of the RP4's to act as chromosome donors may be a reflection of the poor recombination ability of this species (Beringer, 1978). This might be overcome by creating plasmids capable of interaction with the chromosome in a way independent of the recombination system. Boucher *et al.* (1977) and Denarié *et al.* (1977) have used RP4-Mu plasmids for this purpose with some success.

Transfer of only small fragments of chromosome by plasmids (Table 5.1) may mean the conjugative bridge is very fragile in that species, or alternatively that the chromosome fragment is transferred by becoming part of the plasmid (Olsen and Gonzalez, 1974). This would be particularly important in intergeneric crosses where the DNAs of donor and recipient show little ability to hybridise as, for example, in *Pseudomonas* (64% G+C) and *E. coli* (50% G+C). Where such crosses have been tried (Hedges *et al.*, 1977 and Hedges and Jacob, 1977), the plasmids generated are large R's, and one such plasmid is studied in section B.

Chromosome transfer between bacteria is undoubtedly important to the study of genetics as well as in bacterial evolution. Although it may be a rare event in nature, the potential of wide host-range plasmids

to bring about genetic exchange between diverse bacterial genera must have been exploited during the evolutionary time scale.

Although most effort has been put into the study of F owing to its early discovery, it may not be typical. It is certainly unusual in being naturally derepressed for transfer, and also its limited host range may indicate a high degree of specialisation.

Now that chromosome transfer is being used to open up the genetics of other bacterial genera, it begins to look as if there are features unique to every system studied.

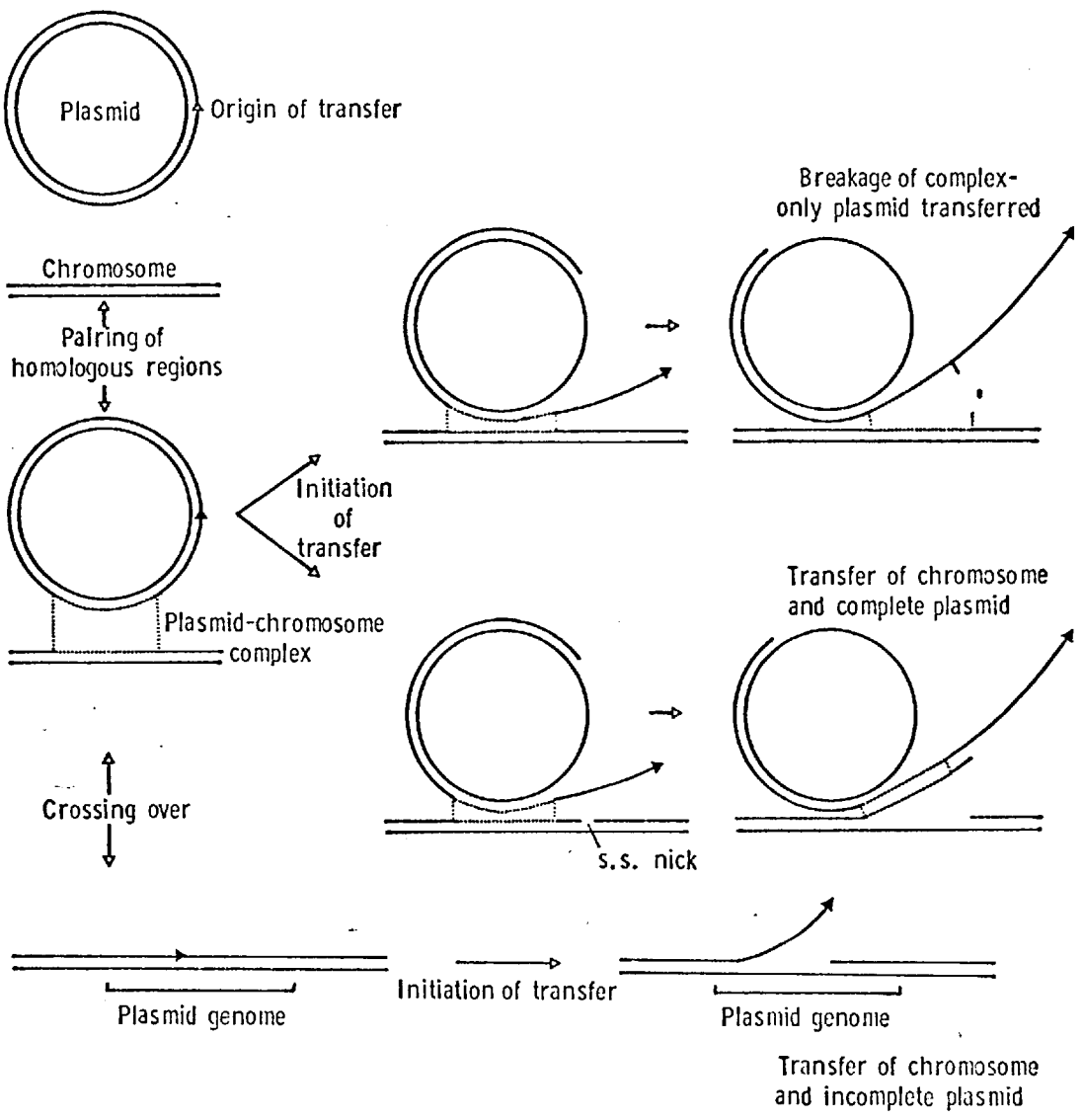
Model for chromosome mobilisation

This model makes two main assumptions:

1. All mobilisation involves interaction between homologous regions on the plasmid and chromosome. These regions may be chromosomal genes, insertion sequences, or both.
2. Insertion of a plasmid into the chromosome to form an Hfr is in two stages which may be mediated by different enzymes:
   i) Recognition and pairing of homologous regions on plasmid and chromosome. This stage is reversible, but occasionally leads to ii) Crossing over between these regions resulting in the insertion of the plasmid into the chromosome.

Stage i) results in the plasmid and chromosome being held together as part of a DNA-protein complex with their homologous sequences in register. If at this stage transfer is initiated by the plasmid, then the complex is broken and only the plasmid transfers unless there is a pre-existing single stranded nick in the chromosome adjacent to the plasmid-chromosome complex. In this case, transfer of the chromosome can originate from this point. This system is illustrated in Figure 5.4. It can explain not only how chromosome transfer can be accompanied by transfer of a complete plasmid, but also how U.V.

Figure 5.4 : Model for chromosome mobilization.



irradiation can stimulate mobilisation by increasing the number of single stranded breaks in the chromosome. Also, the model predicts that Hfr formation is not essential for chromosome transfer. Mutants defective in Hfr formation have been isolated and shown to be still capable of mobilising the chromosome (Curtiss and Renshaw, 1969).

In some organisms, stages i) and ii) may be carried out by the same enzyme, or the first stage may not exist at all, and pairing of homologous regions occurs only by chance. In these cases chromosomal integration of the plasmid is essential for mobilisation to take place.

The reason why some plasmids have no capacity for mobilisation in certain bacterial species is because of a total lack of homology (eg. RP4 in *Rhizobium*, Beringer and Hopwood, 1976). In cases where there is homology between the plasmid and chromosome but still no mobilisation (as described by Jacob *et al.*, 1976) this may be due to the absence of a stage i) mechanism or a reflection of the poor recombinational ability of the host (eg. *Rhizobium*, Beringer *et al.*, 1978). Where plasmids do mobilise in these hosts, it is because they carry structures analogous to insertion sequences which can interact with the chromosome in a manner independent of the recombination system.

The efficiency of the recombination system also influences the frequency with which chromosomal DNA transfer (by whatever mechanism) is detected. In *E. coli* the recombination system may rarely be of importance *in vivo*, and may in fact be an adjunct of or an aberration in a much more important DNA repair process. In other genera, while the system is perfectly competent in repair, the recombinational aspect may be very much reduced. This would affect the frequency with which recombinants can be obtained and also the low frequency of crossing over would mean that large segments of transferred DNA would be incorporated into the recipient chromosome. Conversely, a highly efficient

recombination system would lead to incorporation of multiple small lengths of DNA.

Factors affecting the size of the DNA actually transferred are the stability of the conjugation bridge and whether or not the donor positively terminates transfer. The latter function may be the result of the donor treating the mobilisation event as if it were an intermediate in excision repair and actually cutting the single strand being transferred.

As suggested earlier, mobilisation of the chromosome may be relatively unimportant *in vivo*. Bacteria have evolved towards a minimum genome size so that they can achieve as low a generation time as possible and thus very rapidly colonise new environments. At the same time, they maintain a large genetic pool by means of plasmid-borne characters carried by a small percentage of the population. When selection demands them, these characters can be very rapidly spread by plasmid transfer. In response to new selective pressures (eg. novel antibiotics), genetic material may have to be "drafted" in from other bacterial genera whose DNA is quite unrelated (in terms of G+C ratio) and so would be most unlikely to be incorporated into the chromosome of its new host. In these circumstances, the formation of a suitable plasmid prime in the donor is the only means whereby the information may be transferred.

B. Studies on R68.44p*argF*[1], an R' plasmid generated *in vivo*

R68.44p*argF* was derived from R68.44 by selecting for transfer of arginine independence from *Pseudomonas* to *E. coli* as described in the introduction to this chapter. It carries genes which complement mutations at both the *argF* and *argG* loci in *E. coli* and has a molecular weight of 130 Md (Hedges and Jacob, 1977). Because of its large size it should not be possible to transduce the whole plasmid by bacteriophage P1.

A P1 lysate was prepared from (R68.44p*argF*) 4100, titrated (titre 5×10^9 p.f.u./ml), checked for freedom from bacterial contamination, and used to transduce R^- 4100 (see Chapter 1). Plasmid markers were transduced at a frequency of $1\text{-}5 \times 10^{-5}$ per p.f.u., and 30/30 drug-resistant transductants, whether selected on ampicillin, tetracycline or kanamycin, all had the unselected drug resistances. None were Arg^+, however. Arginine independence was transduced with a frequency of 1×10^{-8} per p.f.u. 30 Arg^+ transductants were purified and tested for antibiotic resistance, transmissibility, and susceptibility to bacteriophage PR4 (Stanisich, 1974) which adsorbs to the tips of the P-1 pili produced by self-transmissible IncP plasmids (Bradley and Cohen, 1977). The results are shown in Table 5.5.

Disregarding the PR4 sensitivities, the Arg^+ transductants fall into four phenotypic classes:

i) 2/30 were $(ApTcKm)^R$ Tra^+ i.e. indistinguishable from clones carrying the parent plasmid.

ii) 8/30 were $(ApTc)^{R_K S}$ Tra^+

iii) 18/30 were $(ApTc)^{R_K S}$ Tra^-

iv) 2/30 were $(ApTcKm)^S$ and did not transfer arginine independence.

---

1 The nomenclature used throughout is that for arginine biosynthesis in *E. coli* (Bachman *et al.*, 1976).

Table 5.5. Arg$^+$ P1 transductants of R68.44*pargF*

Clone no.	Resistances[1]	PR4 Sensitivity[2]	Transmissibility[3]	Clone no.	Resistances[1]	PR4 Sensitivity[2]	Transmissiblity[3]
1	ApTc	S/R	-	16	ApTc	S/R	-
2	ApTc	S/R	-	17	ApTc	S	+
3	ApTcKm	S	+	18	ApTc	R	-
4	ApTc	S/R	-	19	ApTcKm	S	+
5	ApTc	R	-	20	ApTc	R	-
6	ApTc	S/R	-	21	ApTc	S	+
7	ApTc	S	+	22	ApTc	R	-
8	ApTc	R	-	23	ApTc	S	+
9	ApTc	R	-	24	ApTc	S/R	-
10	ApTc	R	-	25	ApTc	S/R	+
11	S	R	-	26	ApTc	S/R	+
12	ApTc	S/R	-	27	ApTc	S/R	+
13	S	R	-	28	ApTc	S/R	-
14	ApTc	R	-	29	ApTc	S/R	-
15	ApTc	S	+	30	ApTc	S/R	-

1 Ap indicates resistance to ampicillin; Tc to tetracycline and Km to kanamycin. S means fully sensitive.

2 Phage tests were done by spotting high titre stocks of PR4 (5×10^9 p.f.u./ml) onto suspensions of plasmid containing strains in soft agar and incubating overnight. (RP4)4100 and R$^-$4100 were +ve and -ve controls. S indicates sensitivity, R resistance and S/R intermediate sensitivity.

3 Transmissibility was tested by plate mating into J53-2, selecting for tetracycline transfer. Complete absence of transfer is scored as -, anything else +. Clones 11 and 13 which were antibiotic sensitive failed to transfer arginine independence to a rifampicin resistant derivative of 4100.

1. Location of the site of insertion of *Pseudomonas* DNA in R68.44.

Assuming: a) Pl packages a single piece of linear plasmid DNA into transducing particles and this piece formed a continuous section of the R68.44*pargF* parent plasmid (consistent with the work of Lee *et al.*, 1974). b) The *Pseudomonas* DNA carried on R68.44*pargF* is a single continuous fragment inserted at a single site on the plasmid.

If these assumptions are valid, the only way the transduction data can be explained is if the *argF* genes and essential R68.44 functions (i.e. plasmid replication) are on a length of DNA small enough to be packed into a Pl phage head. In other words, the *argF* genes are near to one end of the inserted 92.4 Md of *Pseudomonas* DNA.

26/28 of the Arg^+ transductants in which there was co-transduction of R68.44 markers lacked kanamycin resistance, suggesting the site of insertion of the *Pseudomonas* DNA is near the kanamycin gene and that the arginine and kanamycin resistance genes are separated by the essential R68.44 genes.

R68.44 has been shown by heteroduplex analysis to be virtually identical to RP4 (M. van Montagu, personal communication), a genetic map of which has been published (Barth and Grinter, 1977; Barth *et al.*, 1978). Assuming this identity, only two arrangements of R68.44 and the inserted DNA are possible and they are shown in Figure 5.5. The site of insertion has to be between the two *tra* regions (as shown by the double headed arrow) to account for those transductants with a Km^S Tra^+ phenotype.

If arrangement 1 is correct, all transductants which are Arg^+ $(ApTc)^R$ Tra^- will have suffered a deletion of all or part of the transfer region *tra* 1. Similarly, if arrangement 2 is correct, such transductants will be defective in *tra* 2. Using the transductional complementation system described in Chapters 1 and 3, this could be

Figure 5.5: Two possible arrangements for R68.44*argF*.

R68.44 is shown as a nearly complete circle with antibiotic resistance and *tra* genes marked. The inserted *Pseudomonas* DNA is shown roughly to scale with the possible locations of *argF* and regulatory functions. The double-headed arrows show the region within which the insertion must lie (see text).

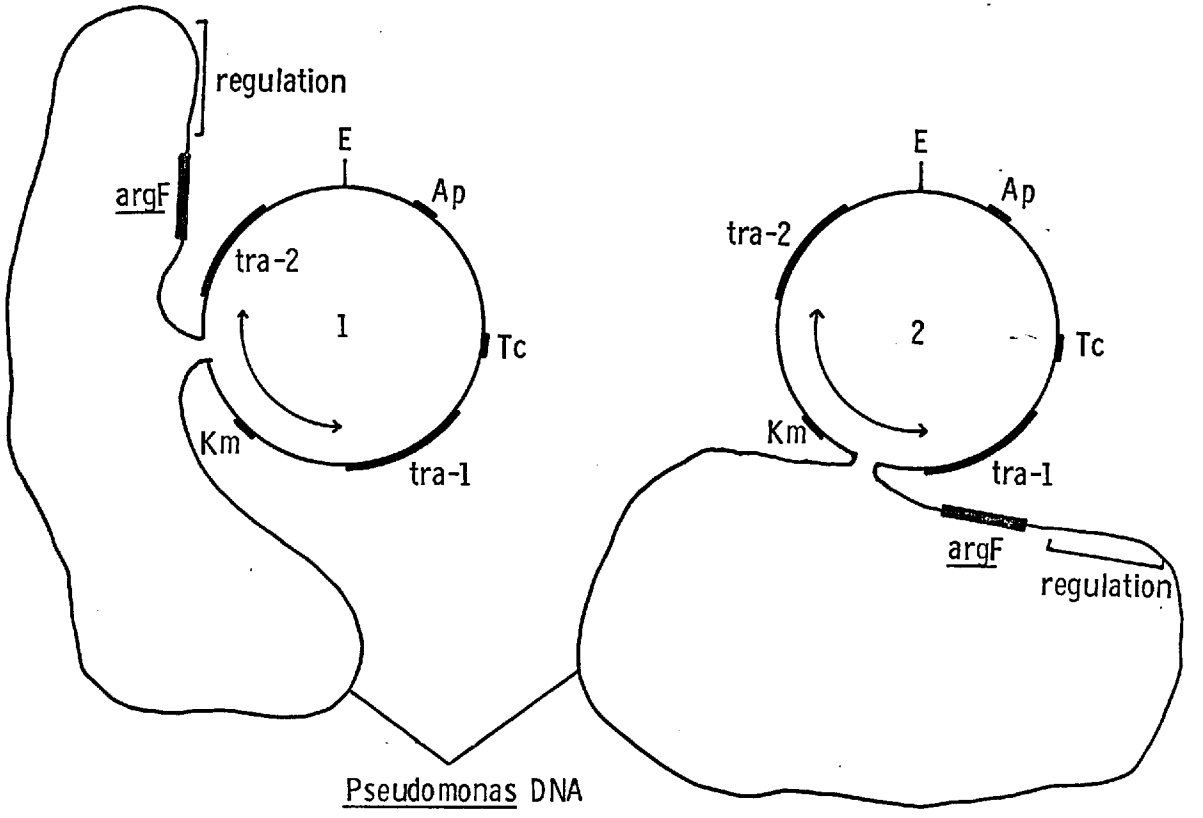


Figure 5.5 : Two possible arrangements for R68.44pargF .

tested. Transient plasmid heterozygotes were formed between clones 5, 22 and 29 (Table 5.5) and derivatives of RP4 which were *tra*$^-$ because of an inserted transposon 7 in either *tra* 1 (pRP82) or *tra* 2 (pRP79). Only pRP82 was able to complement the three Tra$^-$ transductants showing they must have an intact *tra* 1 region and hence that arrangement 2 (Figure 5.5) is the correct one, with the site of insertion between the kanamycin resistance gene and *tra* 1.

2. Compatibility properties of the Arg$^+$ markers in transductant clones 11 and 13. These transductants have no R68.44 markers and do not transfer arginine independence. The Arg$^+$ phenotype was stably inherited through many generations of non-selective growth and the introduction of R702(IncP), selecting for tetracycline resistance, failed to eliminate arginine independence in 20/20 clones tested. Thus the Arg$^+$ character is not on a P plasmid. Introduction of P1CM, however, eliminated Arg$^+$ in 47/48 cases for clone 11, and 48/48 for clone 13 and so the plasmids in these clones are defective P1 prophages carrying *Pseudomonas* arginine genes. Whatever the process of incorporation, the prophage has thereby lost the ability to produce mature phage particles.

3. Properties of transductant clones 3 and 19. These transductants are phenotypically indistinguishable from strains carrying the parent plasmid. All of these characteristics were stably inherited during non-selective growth and all were eliminated by the introduction of R751(IncP), selecting for trimethoprim resistance (20/20 clones tested). P1 lysates were made of these clones and used to transduce strain 4100 R$^-$, selecting for tetracycline or kanamycin resistance or for Arg$^+$. Unlike the parent plasmid, these derivatives now transduced antibiotic resistance and arginine independence with approximately equal frequencies (c. 1×10^{-5} per p.f.u.), indicating that the parent plasmid had

been "transductionally shortened" to a size where the whole plasmid was readily transducible. Because these two plasmids contain most or all of R68.44, it follows that they contain the least *Pseudomonas* DNA of all the transductants (except probably the PlArg plasmids).

4. Testing the transductants for the presence of *argG*. Strain 3050Nal^R *argG lac* Hfr was mated with J53(*pro met*) selecting on lactose plus proline, nalidixic acid and arginine. An *arg pro* MS2^R clone isolated in this way was used as a recipient for all the Tra$^+$ transductant clones listed in Table 5.5. The selection was for tetracycline resistance. Although the parent plasmid R68.44p*argF* was able to complement the *argG* mutation, none of the transductants did so, showing the *argG* and *argF* functions were not closely linked on the *Pseudomonas* DNA carried by R68.44p*argF*.

5. Levels of ornithine carbamoyl transferase produced by the transductants. Ornithine carbamoyl transferase (OCTase) is the enzyme catalysing the conversion of ornithine to citrulline which is the sixth of eight steps (A to H) in the biosynthesis of arginine by *E. coli* (Legrain *et al.*, 1972). The levels of OCTase activity produced by four of the transductants were shown to differ markedly (N. Glandsdorff, personal communication). Transductants 1 and 15 (Ap^{R}Tc^R Tra$^-$ and Ap^{R}Tc^R Tra$^+$ respectively) produced enzyme levels more or less like those determined by the parent plasmid. Transductants 3 (Ap^{R}Tc^{R}Km^R Tra$^+$) and 11(PlArg) however, gave activities 10 and 25 times those produced by the parent. Two possible explanations are i) the transductants producing high OCTase activity are present in more plasmid copies per cell - a gene dosage effect - or ii) there is a *Pseudomonas* gene regulating OCTase production which is missing in transductants 3 and 11, leading to a derepression. The former idea was shown to be incorrect by the measurement of the copy number of clones 1 and 3 (next section). If

there is a regulatory gene on the *Pseudomonas* DNA in R68.44p*argF* somewhere distal to the *argF* gene itself (Fig. 5.5), then its inclusion in a transductant would mean a decreased chance of the whole of R68.44 also being present. The fact that transductants 1 and 15 (normal OCTase) have both lost some R68.44 functions while transuctant 3 (high OCTase) has retained them all is in agreement with this idea.

6. Molecular weights of transductants. ^{3}H labelled CCC DNA was isolated from transductants 1 and 3 by caesium chloride-ethidium bromide density gradient ultracentrifugation (Chapter 1). Cultures of ^{14}C labelled RP4 containing cells were mixed in equal volumes with both transductants before lysis and centrifugation so that excessive shearing or other adverse conditions during the extraction of plasmid DNA would be detected by a reduced recovery of RP4. Copy numbers determined from the ratio of radioactive counts in the plasmid and chromosome bands obtained after centrifugation showed that transductant 1 was present as 0.6 copies per chromosome and transductant 3 as 0.7 copies per chromosome. The values obtained for RP4 from the mixed lysates were 1.7 and 1.9 respectively, falling within the normal range of 1-3. Thus the enhanced OCTase activity produced by transductant 3 (described in the previous section) is not due to an elevated number of plasmid copies.

DNA extracted from the two transductants was subjected to sucrose gradient sedimentation (Chapter 1) and molecular weights of their plasmids determined using the differentially labelled RP4 already present as a molecular weight standard (data not presented). Values of 83 and 90 Md. were obtained for transductants 1 and 3. The unexpectedly large molecular weights of the transductants does not invalidate the arguements presented above, as they make no assumptions about the size of the transduced DNA, only that there is an upper

limit to the length that can be packaged.

Although the P1DNA in phage heads is only 66 Md. and the packaging is believed to be by a "headful" mechanism (Yarmolinsky, 1977), more evidence is presented in the next chapter to show that P1 can transduce plasmids of up to 90 Md. molecular weight.

Summary of results

1. P1 transduction of the plasmid R68.44p*argF* gave Arg^+ transductants which had retained all, some, or none of the R68.44 markers. Analysis of the patterns of markers obtained plus data from complementation of RP4 *tra* mutants allowed the site of insertion of *Pseudomonas* DNA into R68.44 to be located.

2. Two transductants lacking any R68.44 markers were shown to carry arg^+ genes in defective P1 prophages.

3. Two transductants with markers phenotypically indistinguishable from those determined by the parent plasmid were shown to carry plasmids that had undergone transductional shortening.

4. Unlike the parent plasmid, none of the self-transmissible plasmids in the transductants had the ability to complement mutants in *argG*.

5. Two transductants gave elevated levels of ornithine carbamoyl transferase activity. The presence of a *Pseudomonas* regulatory gene on R68.44p*argF* was inferred.

6. Molecular weight measurements on two transductants showed them to be more than 30% larger than the amount of P1 DNA normally packaged in a phage head.

Discussion

R68.44 mediated intergeneric transfer of chromosomal DNA provides a means for studying the expression of bacterial genes in novel genetic backgrounds. It also allows the genetic organisation of the donor to

be analysed. P1 transduction was used to "pare down" the chromosomal material carried on the plasmid and has allowed separation of the *Pseudomonas argF* and *argG* genes. In some cases this has led to derepression of the *argF* gene, probably by deletion or inactivation of a *Pseudomonas* control gene.

The observations that a bacteriophage can transduce large (> 80 Md) molecular weight DNA and that the *argF* genes from *Pseudomonas* can be incorporated into a defective prophage, both show that phages in general may have an important role in genetic transfer between diverse bacterial species. Although R factor transfer has been demonstrated in nature between certain enteric bacteria (Kagiwada *et al.*, 1960), other genera in different environments may rely much more on phages as agents in genetic exchange. Some of the intricate network of pathways that exist for phage transfer between soil organisms are presented in a review by Reanney (1976) and the question is further discussed in the next chapter.

General Discussion: Phage and plasmid origin

With increasing knowledge, rigid ideas about bacterial speciation have to change. The variety of mechanisms for genetic interchange already known means that there are virtually no barriers to the dissemination of genetic information. Plasmids and phages are very important agents in this spread since any extrachromosomal replicon, once established, can acquire by transposition, recombination, etc. new characters which are manifested in response to selective pressure. The problem of their origin remains, however. Clearly they cannot have existed before bacteria and may have been formed degenerately from, say, organelles of Protozoa.

Alternatively, plasmids and phages may have evolved from bacteria themselves. Leavitt *et al.* (1971) showed that in Hfr mediated crosses from *E. coli* to *Salmonella*, covalently closed circular DNA could be isolated from some recipients. No F functions due to these molecules could be detected. Consideration of the selections used makes it likely the plasmids generated carry the *E. coli* origin of chromosomal replication which has recently been mapped between *phoS/bglR-rbs* (Fayet and Louarn, 1977).

The origin of replication and its surrounding DNA could therefore be a latent extrachromosomal replicon. This idea could also explain in part the host ranges of plasmids. A plasmid will not be stably inherited in a host whose chromosome is subject to the same replication controls as itself. In other words, the two would be incompatible. Some evidence for this kind of instability was found by Hiraga (1976) who studied selected F's by their ability to replicate in Hfrs. These plasmids all carried the region of chromosome around the origin of replication and were somewhat unstable.

Only further research on the biochemistry of plasmid and chromosome replication control will show if these ideas have any foundation in fact.

CHAPTER 6

PHAGES AS AGENTS IN PLASMID SPREAD AND EVOLUTION

Abstract

The bacteriophage P1 was used to transduce a plasmid which was considerably larger than the phage genome. The transductants exhibited a spectrum of properties, including some which were phenotypically indistinguishable from the parent plasmid, and one which was a P1 prophage carrying genes for tetracycline resistance. Consideration of the patterns of markers transduced allowed a tentative genetic map to be drawn. Further genetic analysis was carried out including a test on the effect of the *recA* locus in the transductional recipient. Molecular weight measurements on the transductants showed the plasmids to range from about 45-90 Md. Analysis of the plasmid DNAs by restriction enzyme cutting and gel electrophoresis was used to characterise the transductants, locate certain genes and essential regions, relate the genetic and physical maps, and investigate the packaging of plasmid DNA by P1. Mechanisms of P1 transduction are discussed.

Introduction

Bacteriophages are associated with virtually all naturally occurring bacterial populations. They are found in a variety of different states: they may be present as free phage particles capable of infecting a recipient cell, causing lysis, lysogeny, transduction or conversion; they may exist as lysogens inside the cell with most phage functions repressed, replicating in synchrony with the host either as part of the chromosome or as a plasmid; they may, like the filamentous phages, cause the host to release mature phage particles during its growth without appreciably harming it (Marvin and Hohn, 1969); finally, they may be grossly defective or cryptic, revealing their remaining phage-like characters only after chemical induction and producing only transducing particles (Huang and Marmur, 1970).

Among coliforms, nearly 100 coliphages have been documented, including 30 different prophages (Reanney, 1976). Many diverse types are known, and although their ecology has been little studied, their ready isolation from sewage anywhere in the world suggests that all natural coliform populations harbour them. In the genus *Pseudomonas* the frequency of lysogenic strains has been put as high as 100%, with polylysogeny occurring (Holloway and Krishnapillai, 1975). Bacteriophages have also been identified in gram positive organisms such as *Staphylococcus aureus* (Novick and Morse, 1967), and in *Bacillus* over 150 phages have been reported (Reanney, 1976). These examples show some of the range and diversity of phages. Calculations based on the number of viruses found in soil samples show them to be the most numerous genetic objects by several orders of magnitude (Reanney, 1975) and if, as Szybalski and Szybalski (1974) believe, the chromosomes of all organisms contain viral "modules" that may occasionally acquire replicative autonomy, then it follows they will be universally distri-

buted throughout the biosphere.

While the principal function of phages is to ensure their own survival, they can also be responsible for alterations in the properties of their hosts. Examples of this are: lysogeny conferring superinfection immunity, phage mediated modification and restriction systems (Glover *et al.*, 1963), and even enhanced growth rate under certain conditions (Lin *et al.*, 1977; Edlin *et al.*, 1977); converting phages affecting characters such as antigenicity (in strains of salmonellae, Robbins *et al.*, 1965) or pathogenicity (production of diptheria toxin in *Corynebacterium diptheriae*, Gill *et al.*, 1972); transducing phages, transferring plasmid or chromosomal genes, whole plasmids and even other phages. This latter property may be especially important for genetic transfer in genera of gram positive organisms where conjugative plasmids are less common. This has been demonstrated in staphylococci (Novick and Morse, 1976; Inoue and Mitsuhashi, 1975). It may well be that the habitat of many gram positive organisms may militate strongly against genetic transfer by conjugation. In the soil, for example, which is a colloidal and particulate medium, the variability of such factors as nutrient availability, temperature and pH, may mean that the soil bacteria are seldom present in adequate numbers in sufficient proximity and in a suitable metabolic state to permit an efficient transfer of information by conjugation. In these circumstances, natural selection may well have acted to promote cell to cell transfer by smaller, more diffusible and dispersible phage particles.

Another important aspect of genetic transfer by transduction is that it allows interspecific exchanges of information. For example, there is such an enormous potential for phage mediated interchange of genes in the genus *Bacillus* that it may constitute a single genospecies (Reanney and Teh, 1976). Among coliphages, too, there are those with

wide host range, particularly among those mediating generalised transduction. P1, for example, infects not only *Escherichia* spp., but also *Salmonella*, *Shigella* and *Klebsiella*. Kaiser and Dworkin, 1975, have shown it can transfer genes from *E. coli* to myxobacterium.

Regarding the role of phages in plasmid transfer, they may be the only means of bringing it about in some species and in certain environments (eg. *Bacillus*). In other cases, they may expand the potential host range of plasmids in several ways: phage-mediated modification systems allow plasmids to infect lysogenic strains in which they would otherwise be restricted (Glover *et al.*, 1963); certain interspecific and intergeneric plasmid exchanges may be exclusively phage-mediated due to the lack of suitable conjugation systems; transduction can reduce the size of plasmids (Pittard and Adelberg, 1963) thereby deleting sites for restriction enzymes and genes which might be harmful to the new host (such as genes for pilus production, causing sensitivity to male-specific phages).

Phages can also incorporate transposons (Kondo and Mitsuhashi, 1964) and chromosomal genes (Rosner, 1975 and this thesis, Chapter 5) into their genome, the new material replacing redundant DNA and non-essential phage genes when packaged in the phage head (Yun and Vapnek, 1977), resulting in these new characters being transduced into the recipient as part of a plasmid prophage. If the inserted DNA is smaller than the redundant DNA normally packaged, then the phage is still capable of lysing its host and releasing infective particles which transduce the incorporated material at high frequency.

In addition to involvement in the transfer and evolution of existing plasmids, phages may also create new plasmids like those described by Leavitt (1971) and Hiraga (1976) by transduction of the chromosomal origin of replication of their hosts. The question of plasmid evolution is discussed further in Chapter 5.

Bacteriophage P1

1. Molecular properties. DNA from infective P1 phage particles is linear, double stranded, circularly-permuted and has a molecular weight of about 66 Md. (Ikeda and Tomizawa, 1968), and with extensive terminal redundancy (9-12% of the genome, Yun and Vapnek, 1977). In the prophage state, P1 is maintained as a circular plasmid molecule without redundancy, at one to two copies per chromosome and a molecular weight of 58 Md. (Hedges *et al.*, 1975). DNA from the closely related phages P1Cm and P7 (formerly ØAmp) is the same size as linear P1 but with reduced redundancy, and their prophages are correspondingly larger (Hedges *et al.*, 1975; Yun and Vapnek, 1977).

In addition to the normal sized particles, about 25% of a population of P1 phages are smaller, the majority of the class containing about 40% of the DNA of the large particles (Ikeda and Tomizawa, 1965c). These particles are defective, but can inject their DNA and phage markers can be rescued from mixed infections (Walker and Anderson, 1970).

2. Genetics of P1. This has been extensively studied by the isolation, characterisation and mapping of mutants, initiated by Scott, 1968. About 120 amber mutants in functions required for plaque formation have been isolated and put into ten linkage groups (Walker and Walker, 1976). Mutants affecting lysogeny, immunity, restriction, modification, a dnaB-like function, lysis and transduction have also been isolated (see Yarmolinsky, 1977).

Much work has been done on the immunity to superinfection and the maintenance of lysogeny by the P1 prophage. In λ, both functions are mediated by the same repressor protein, the product of the *cI* gene (Echols and Murialdo, 1978). In P1, however, the functions are separable (Scott, 1975). Further, although P1 and P7 have different immunities (Chesney and Scott, 1975) their prophages behave like

incompatible plasmids (Hedges *et al.*, 1975) indicating a common mechanism of replication control. In P1 and P7 identical *cI* products negatively control prophage replication while another gene, *c4*, controls immunity. The *c4* products which are different in the two phages act by repressing *ant*, an antirepressor which acts against *cI*. *ant* is not required for lytic growth or lysogeny but may be preserved because it allows a readily reversible transition to virulence by a single step mutation to *ant* constitutive (Wandersman and Yarmolinsky, 1977).

Studies by Rosner, 1972, showed that P1 lysis, lysogeny, spontaneous curing and production of transducing particles all took place in a *recA* background. The possible existence of a P1 specified generalised recombination system was demonstrated by Hertman and Scott, 1973. Recombination between P1 mutants was observed at reduced frequency in *recA*, *recB* and *recAB* genetic backgrounds.

3. Specialised transduction. The ability of P1 to incorporate foreign DNA into its genome and still lyse its host was first demonstrated by Kondo and Mitsuhashi (1964). They obtained a recombinant phage, P1Cm, which carried genes for chloramphenicol resistance from an R factor. Its phage functions were unimpaired and it transduced chloramphenicol resistance at high frequency.

Takano and Ikeda (1976) developed a selective technique for isolating such recombinants, producing a P1Km phage. Rosner (1975) transduced a *recA* strain and isolated a P1-*pro*$^+$ phage. On induction, this gave a mixture of products, some of which were defective phages which transduced *pro*$^+$ at high frequency, and some were plaque-forming particles (10% of the normal yield by P1). It was presumed that the P1-*pro*$^+$ prophage was oversized and could not be completely incorporated into phage heads. (This does not explain its formation, however.)

This is an example of the general observation of an upper limit to the size of DNA which can be inserted into the phage genome without loss of phage functions from any particles subsequently formed. Takano and Ikeda (1976) showed this was true for P1KmCm, as did Mise and Arber (1976) for P1Tc and P1CmTc, and Iida and Arber, 1977, for P1CmSmSu.

Restriction enzyme cleavage of phage DNA (Schulz and Stodolsky, 1976) and heteroduplex analysis (Yun and Vapnek, 1977) were used to locate the insertions in several specialised transducing derivatives of P1. The heteroduplexes showed the incorporated material appeared in the phage heads instead of some of the redundant phage DNA. If the foreign DNA was bigger than the redundancy (c 5 Md.) then phage particles contained incomplete P1 genomes. This suggested that the mechanism of DNA packaging was like the "headful" hypothesis put forward by Streisinger *et al.* (1967) for phage T4. This is disucssed in the next section.

4. Generalised transduction. P1 is arguably the best studied generalised transducing phage. Lennox (1955) demonstrated that it could transduce a wide variety of markers and it is now beleived that in a population of P1 phage, the transducing particles contain randomly distributed host DNA fragments. Although the transduction frequency varies for different markers due to differences in efficiency of expression or incorporation, for a given marker the transduction frequency is proportional to its gene dosage at the time of cell lysis. This has been used in experiments to locate the origin of replication of the *E. coli* chromosome (Caro and Berg, 1971), the nearer a marker to the origin, the greater will be its gene dosage in an exponentially growing cell population.

P1 transduction has also been particularly useful in the mapping of the *E. coli* chromosome (Bachman *et al.*, 1976) and in the analysis of

R factor structure (Kondo *et al.*, 1962).

The molecular nature of the transduction process was first investigated in a series of papers by Ikeda and Tomizawa (1965a, b and c). Using differential radioactive and density labelling they were able to separate transducing particles from normal P1 phage. They concluded that transducing particles formed 0.3% of the total phage population, and that they contained bacterial DNA only which was of the same molecular weight as the P1 DNA normally packaged. About 25% of the phage were small, containing only 40% as much DNA as the large particles. Small transducing particles were also detected and shown to carry a similarly reduced amount of bacterial DNA. Such phage were incapable of transducing markers as widely separated on the chromosome as the large P1 transducing phage. (P1 minute particles have since been detected, Walker and Anderson, 1970.) These results supported the idea that for both P1 and host cell DNA, contiguous headfuls were packed rather than the DNA being cut by an enzyme acting as a molecular ruler.

The headful hypothesis of Streisinger *et al.* (1967) was put forward to explain the nature of the DNA found in the heads of T4 phage. According to their model, T4 DNA was synthesised as a long concatamer and used to fill phage heads which hold rather more than one complete genome. This would explain the observation of both circular permutation and terminal redundancy in T4 DNA. A prediction of this model is that deletion of part of the phage genome would not affect the length of DNA per phage, but would increase the redundancy. This was true for T4 (Streisinger *et al.*, 1967) and the work of Yun and Vapnek (1977) strongly suggests the same mechanism operates in P1.

Further evidence was provided by Lee *et al.*, 1974, who used heteroduplex analysis to compare P1 transductants of a large F' (F14)

with the parent plasmid. Their experiments showed the transductants were about the same size as P1, contained no P1 DNA and the transduced sequences were homologous with continuous stretches of the parent plasmid (except for one which had an 800 kilobase insertion, possibly IS1).

Packaging of host DNA by P1 is not the result of a phage mutation. This was demonstrated by Harriman (1972) who showed that both transducing and lytic phage particles could be liberated in the same burst. In addition, 0.5% of P1 bursts produced particles capable of transducing λ from the lysogenic donor. Other P1 particles from the same burst were frequently able to transduce another lysogenic phage from a widely separated site on the donor chromosome. The frequency of co-production of such particles was too high to be accounted for by random packaging of the host DNA (although it was related to the distance between the lysogenic phages). A model was proposed suggesting that there were regions in which chromosomal DNA was efficiently packaged in a proportion (c 10%) of the hosts.

Wall and Harriman (1974) used hydroxylamine mutagenesis to isolate mutants of P1 with altered transduction frequencies. Both high and low frequency transducing mutants were isolated, the frequency being altered for all markers, but not equally. The burst size was normal and the mutants non-complementing. The authors suggested that a P1-coded nuclease specific for P1 DNA had altered affinity for bacterial DNA in the mutants. Consistent with this was the accelerated breakdown of host DNA reported for one of the high frequency transducing mutants (Harriman and Wall, 1973).

Finally, there is evidence that P1 packaging begins at preferred sites, as is the case for phage P22 (Tye *et al.*, 1974). When cleavage fragments from restriction enzyme digests of DNA from P1 particles are

compared by electrophoresis with those produced from the P1 prophage, an additional band is observed in the phage digests. The new band contains material from the right hand end of the phage molecule as it is conventionally drawn. Also, densitometry measurements showed bands originating from the region immediately to the right of the extra band were more intense in phage digests than those from prophage DNA. This was interpreted as meaning that packaging was initiated at a specific site near the right hand end of the P1 molecule and packaging continued to the right of this site. The termination point was determined by an imprecise "headful" mechanism, explaining why only one clearly defined new band was seen in the phage digests. In addition, the intensity of the novel fragment indicated in contained one third as many molecules as the other bands. This means that if P1 is packaged from concatameric molecules, the average number of sequential packaging events is three (Bächi and Arber, 1977).

If an enzyme is responsible for the generation of the packaging origin, it may also initiate packaging of chromosomal DNA from specific sites (explaining the results of Harriman, 1972). When mutant, this enzyme could alter the incidence of transducing particles (Wall and Harriman, 1974).

Results

RA1-1 is a plasmid of incompatibility group A of molecular weight 90 Md. (P. T. Barth, unpublished). It was isolated as a recombinant between RA1 (IncA, $Tc^{R}Su^{R}$) and R57b (IncC, $Ap^{R}Cm^{R}Su^{R}Gm^{R}Km^{R}$) and carries genes for resistance to chloramphenicol, tetracycline and sulphonamides (Datta and Hedges, 1973). It was reported that the resistances of RA1-1 were co-transducible by P1 (Datta and Hedges, 1973) and the molecular weight of one such transductant was about 90 Md. (Barth and Grinter, unpublished).

A P1 lysate was prepared from J62(RA1-1), titrated (titre 4.4×10^{9} p.f.u./ml after dilution in P1 buffer), and checked for the absence of bacterial contamination. 0.3 ml of undiluted phage lysate was plated onto nutrient agar and incubated at 37°C overnight. No growth was observed.

J53-2 was transduced with the lysate at a multiplicity of infection (m.o.i.) of 2, and plated on minimal agar containing the requirements of J53-2 plus rifampicin and either chloramphenicol or tetracycline. Controls of J53-2 alone were plated on the same media. After 48 hours incubation, no growth was observed on the controls, whereas colonies appeared on both chloramphenicol and tetracycline selections, corresponding to transduction frequencies of 2×10^{-8} and 1×10^{-7} per p.f.u. respectively. 24 colonies from each selection were purified on the same selective media. These were called Cm1-24 and Tc1-24. Each isolate was tested for unselected drug resistance markers, transmissibility and surface exclusion. The results are shown in Tables 6.1(a) and (b).

With the exception of Tc1 and Tc19 which are discussed later, the pattern of phenotypes exhibited by the transductants is consistent with the arrangement of genes on RA1-1 as shown in Figure 6.1. The

Table 6.1 (a). Properties of RA1-1 transductants

Plasmid	Resistance[1] pattern	Surface[2] exclusion	Transmissibility[3]	Plasmid	Resistance pattern	Surface exclusion	Transmissibility
Cm1	CTSu	+	+	Tc1	TSu	+/-	-
Cm2	CTSu	+/-	-	Tc2	CTSu	-	-
Cm3	CTSu	+	-	Tc3	CTSu	+	+
Cm4	CTSu	+	-	Tc4	TSu	-	-
Cm5	CTSu	+/-	-	Tc5	TSu	-	-
Cm6	CTSu	+	+	Tc6	TSu	-	-
Cm7	CTSu	+/-	-	Tc7	CTSu	+/-	-
Cm8	CTSu	+	-	Tc8	CTSu	-	-
Cm9	CTSu	+	-	Tc9	TSu	-	-
Cm10	CTSu	+/-	-	Tc10	TSu	+	+
Cm11	CTSu	+/-	-	Tc11	TSu	-	-
Cm12	CTSu	+	-	Tc12	CTSu	+/-	-
Cm13	CTSu	+	-	Tc13	CTSu	-	-
Cm14	CTSu	+/-	-	Tc14	CTSu	-	-
Cm15	CTSu	+	-	Tc15	TSu	-	-
Cm16	CTSu	+/-	-	Tc16	CTSu	-	-
Cm17	CSu	+/-	-	Tc17	CTSu	+	-
Cm18	CTSu	+	-	Tc18	CTSu	-	-
Cm19	CTSu	+/-	-	Tc19	T	+	defective (10% normal)
Cm20	CTSu	+/-	-	Tc20	CTSu	+	+
Cm21	CTSu	+/-	-	Tc21	TSu	-	defective (3% normal)
Cm22	CTSu	+	-	Tc22	TSu	+	+
Cm23	CTSu	+/-	-	Tc23	CTSu	-	-
Cm24	CTSu	+	+	Tc24	CTSu	-	-

Footnotes to Table 6.1 (a)

1 Antibiotic resistances are: C - chloramphenicol; T - tetracycline; Su - sulphonamides.

2 The ability to exclude the entry of R40a (Datta and Hedges, 1973) was measured. Overnight broth matings were carried out between J62(R40a) and the transductants in J53-2. J53-2(RA1-1) and J53-2(R^-) recipients were used as positive and negative controls respectively. Selection was for kanamycin resistance on isosensitest medium with rifampicin to counterselect the donor. Typically there were less than 10 colonies on the positive controls and between 5000 and 10,000 on the negative controls. Scoring was as follows: Sex^+ - no. of colonies < 10 times +ve control; Sex^- - no. of colonies > 10% of -ve control; $Sex^{+/-}$ - no. of colonies > 10 times +ve control, but < 10% of -ve control.

3 Transmissibility was tested in overnight broth matings with J62-1 as the recipient. Selection was for chloramphenicol (Cm1 - Cm24) or tetracycline (Tc1 - Tc24) resistance with nalidixic acid to counterselect the donor. (RA1-1)J53-2 and (R^-)J53-2 were positive and negative controls, respectively. Typically, there were no colonies on the negative controls and over 1000 on the positive controls. With the exception of Tc19 and Tc21, transfer was either indistinguishable from RA1-1 or undetectable.

Table 6.1 (b). Properties of RA1-1 transductants

Phenotypic class	Number of representatives[1]	Clone numbers
1. CTSu $Sex^{+/-}$ Tra^{-}	13/48	Cm2,5,7,10,11,14,16,19,20,21,23,Tc7,12
2. TSu Sex^{-} Tra^{-}	6/24	Tc4,5,6,9,11,15
3. CTSu Sex^{+} Tra^{-}	10/48	Cm3,4,8,9,12,13,15,18,22,Tc17
4. CTSu Sex^{-} Tra^{-}	8/48	Tc2,8,13,14,16,18,23,24
5. CTSu Sex^{+} Tra^{+}	5/48	Cm1,6,24,Tc3,20
6. TSu Sex^{+} Tra^{+}	2/24	Tc10,22
7. CSu $Sex^{+/-}$ Tra^{-}	1/24	Cm17
8. TSu Sex^{-} $Tra^{defective}$	1/24	Tc21
9. TSu $Sex^{+/-}$ Tra^{-}	1/24	Tc1
10. T Sex^{+} $Tra^{defective}$	1/24	Tc19

1 The phenotypic classes are listed in descending order of frequency of occurrence. Chloramphenicol sensitive or tetracycline sensitive derivatives could not be selected on these antibiotics and so their numbers are expressed as a fraction of 24, rather than 48.

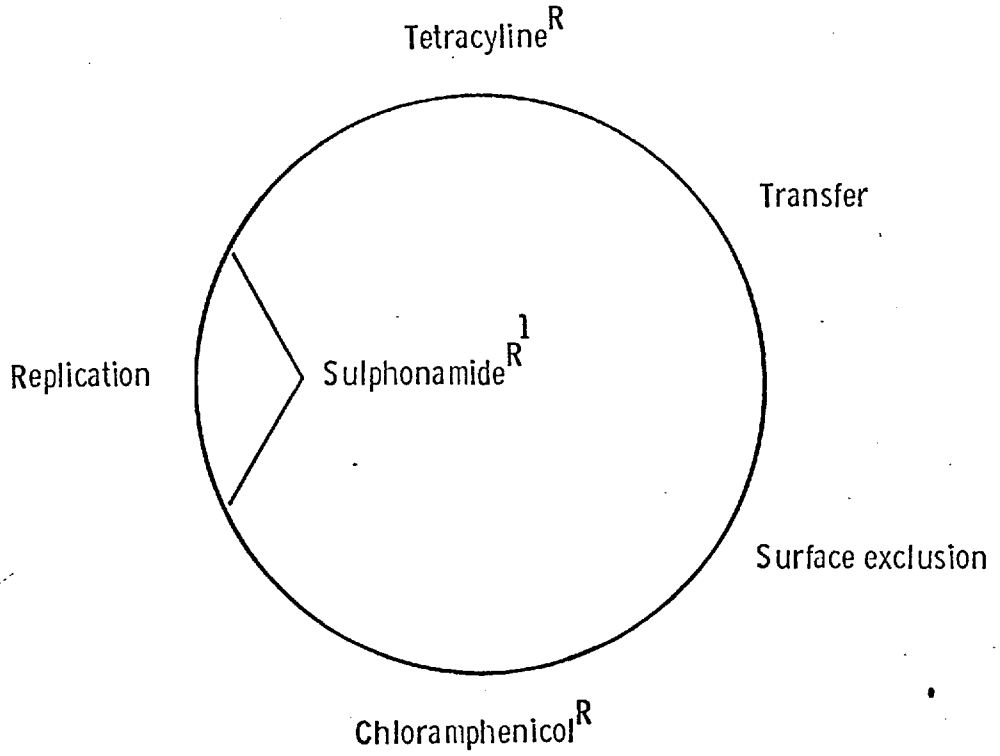


Figure 6.1: Map of RA1-1 derived from transduction data.

The spacing of markers around the circular map is based on aesthetic considerations. No conclusions about intervals between genes can be drawn from the transduction data (see text).

[1]The two possible locations of the sulphonamide resistance gene(s) are shown.

data say nothing about the distances between markers since each selection demands the inclusion of the selected antibiotic resistance, the essential RA1-1 genes and the material in between. Hence, frequency of co-transduction is a function both of the size of the piece of RA1-1 demanded by the selection as well as the distance between selected and unselected markers. Also, data presented later in this chapter show that the length of the transduced DNA may vary, introducing another unknown into the relationship.

One puzzling feature of the results is the failure of chloramphenicol selection to produce a Sex^- phenotype, while 8/24 of the tetracycline selected clones are $C^R Sex^-$.

Transduction of a *recA* recipient. DNA isolated from phage particles is linear (Ikeda and Tomizawa, 1964; Bächi and Arber, 1977; Yun and Vapnek, 1977). When plasmids are transduced, what mechanism allows them to recircularise in the new host? To test the involvement of the *recA* gene in this process, cultures of C600 and HH26 (C600*recA*) were transduced under identical conditions with the phage lysate from RA1-1 described above. The results are shown in Table 6.2

Transduction of a chromosomal marker (*leu*$^+$) was undetectable into the *recA* recipient. The tetracycline resistance from RA1-1 was transducible to C600*recA*, however, with a frequency 25% of that for the isogenic *recA*$^+$ strain. Allowing for the reduced viability of *recA* cultures, this does not represent a significant difference and shows the *recA* gene is not required in this process. Four tetracycline resistant colonies from each recipient were purified and tested for antibiotic resistance. 3/4 from the *recA*$^+$ strain were TcSu resistant, 1/4 was CmTcSu resistant. 4/4 of the *recA* transductants were TcSu resistant. The results could be due to a TcSu transposon, but this is considered unlikely because of the molecular studies described below.

Table 6.2. Transduction of *recA*$^+$ and *recA* recipients

Donor strain	Transductional recipient	m.o.i.	Transduction frequency (per p.f.u.) Tc resistance	*leu*$^+$
(RA1-1)J62	C600	0.5	4×10^{-7}	2×10^{-6}
(RA1-1)J62	C600*recA*	0.5	1×10^{-7}	$< 5 \times 10^{-9}$

There was no growth from platings of the recipients alone (no P1) on the same selective media.

DNA was prepared from 24 of the transductants described in the previous section and of these, five had a phenotype consistent with their having received a chromosomally inserted TcSu transposon and no plasmid (i.e. they were $Tc^{R}Su^{R}$ $Sex^{-}Tra^{-}$). 5/5, however, were shown to contain plasmids related to the RA1-1 in the donor (see below). There is therefore no evidence for a TcSu transposon.

Another possibility is that the RA1-1 plasmid spontaneously produces segregants which are chloramphenicol sensitive and small enough to be efficiently transduced in supercoiled form. A search for such segregants by replica plating failed to show any in approximately 10^{4} colonies tested. It therefore is strongly suggested that the $Tc^{R}Su^{R}$ transductants observed in the *recA* recipient are the result of the packaging of segments of RA1-1 into phage particles. The resulting linear plasmid fragments reform circular molecules in the new host by a *recA* independent mechanism.

Properties of Tc19. This transductant was unusual in two respects: firstly, it was the only RA1-1 derivative lacking sulphonamide resistance; secondly, molecular studies (Plates 6.3 and 6.8) showed it to contain a plasmid quite unrelated to the parent plasmid. One possibility was that the tetracycline genes from RA1-1 had become part of a P1 prophage. Accordingly, its compatibility properties were tested against RA1-1b(IncA) and P1Cm(IncY), with two other transductants (Tc10 and Tc11) included as controls. The results are shown in Table 6.3 and indicate that Tc19 is incompatible with P1Cm and compatible with RA1-1b. Tc10 and 11 both behaved like IncA plasmids.

The ability of P1 to form plaques on Tc19, Tc10 and Tc11 was also tested (Table 6.3) and the results indicate clearly that Tc19 expresses P1 immunity. This result and the previous one suggest very strongly that the plasmid in Tc19 is a P1 prophage. However, Tc19 did show a

Table 6.3. Properties of Tc10, Tc11 and Tc19

Donor	Recipient	Compatibility	Exclusion (colony counts)	P1 immunity (plaque counts) 10^{-7}	10^{-8}	
(RA1-1b)J62	(Tc10)J53-2	24/24 $Cm^{R}Tc^{S}$ Incompatible	74 (Sex^{+})			
(RA1-1b)J62	(Tc11)J53-2	24/24 $Cm^{R}Tc^{S}$ Incompatible	> 1000 (Sex^{-})			
(RA1-1b)J62	(Tc19)J53-2	24/24 $Cm^{R}Tc^{R}$ Compatible	> 2000 (Sex^{-})			
(RA1-1b)J62	J53-2		c 5000 (Sex^{-})			
(P1Cm)W3110T^{-}	(Tc10)J53-2	24/24 $Cm^{R}Tc^{R}$ Compatible	c 5000 (Sex^{-})			
(P1Cm)W3110T^{-}	(Tc11)J53-2	24/24 $Cm^{R}Tc^{R}$ Compatible	c 5000 (Sex^{-})			
(P1Cm)W3110T^{-}	(Tc19)J53-2	24/24 $Cm^{R}Tc^{S}$ Incompatible	c 2000 (Sex^{-})			
(P1Cm)W3110T^{-}	(R40a)J62	12/12 $Cm^{R}Km^{R}$ Compatible	c 5000 (Sex^{-})			
(P1Cm)W3110T^{-}	J53-2		c 5000 (Sex^{-})			
	(Tc10)J53-2			> 500	75	(Imm^{-})
	(Tc11)J53-2			> 500	90	(Imm^{-})
	(Tc19)J53-2			0	0	(Imm^{+})
	(RA1-1)J53-2			> 500	50	(Imm^{-})
	(P1Cm)W3110T^{-}			0	0	(Imm^{+})
	J53-2			> 500	80	(Imm^{-})

Table 6.3 Continued. Properties of Tcl0, Tcl1 and Tcl9

Donor	Recipient	Compatibility	Exclusion (colony counts)	Pl immunity (plaque counts)
(R57b-1)J62	(Tcl0)J53-2	23/23 Cm^RTc^S Incompatible	62 (Sex^+)	
(R57b-1)J62	(Tcl1)J53-2	24/24 Cm^RTc^S Incompatible	> 1000 (Sex^-)	
(R57b-1)J62	(Tcl9)J53-2	22/24 Cm^RTc^R: 2/24 Cm^RTc^S Compatible?	c 5000 (Sex^-)	
(R57b-1)J62	J53-2		c 5000 (Sex^-)	
(R40a)J62	(Tcl0)J53-2	20/20 Km^RTc^S Incompatible	c 400 (Sex^+)	
(R40a)J62	(Tcl1)J53-2	Not tested	c 5000 (Sex^-)	
(R40a)J62	(Tcl9)J53-2	11/21 Km^RTc^R: 10/21 Km^RTc^S	c 500 (Sex^+)	
(R40a)J62	(RAl-1)J53-2	Not tested	118 (Sex^+)	
(R40a)J62	(PlCm)J53-2	5/9 Km^RCm^R: 4/9 Km^RCm^S	c 500 (Sex^+)	
(R40a)J62	J53-2		c 10^4 (Sex^-)	

marked ability to exclude the entry of R40a (Table 6.1). To confirm this it was retested against RA1-1b, R57b-1 [IncC, but excluded strongly by IncA plasmids (Datta and Hedges, 1977a)] and P1Cm (Table 6.3). No evidence of exclusion was found. On retesting with R40a, however, its exclusion by Tc19 was confirmed and it was also shown that P1Cm had the same ability to exclude R40a. This last observation suggests that Tc19 has not acquired its ability to exclude R40a from RA1-1.

The compatibility properties of P1Cm, Tc19 and R40a were also tested. Whereas P1Cm was not excluded by and did not eliminate R40a, elimination of Tc19 and P1Cm by R40a was observed in approximately 50% of the transfers (Table 6.3). When clones harbouring both the Km^R of R40a and the Tc^R of Tc19 were retested after growth for several generations without selection, they were found to have retained both resistances (and P1 immunity) in all cases (16/16). The tetracycline resistance of these stable "doubles" was separately transmissible, but all attempts to transfer the kanamycin resistance failed.

The results are extremely difficult to interpret. That Tc19 is a P1 type plasmid (IncY) has been clearly demonstrated as has the membership of Tc10 and Tc11 to IncA. The interactions of R40a with P1Cm and Tc19, however, do not fit into any published description of plasmid behaviour.

A hypothesis to explain the dislodgement of one plasmid by another compatible plasmid was suggested by Coetzee *et al.*, 1975. In this model, genes for a hypothetical nuclease are transiently induced when the plasmid carrying them enters a new host by conjugation. This enzyme specifically attacks the resident plasmid, causing its loss or the deletion of a particular section of it. In the results presented here, the one case where a plasmid may have undergone a specific deletion (the loss of transmissibility of R40a from a "double" with Tc19)

it was the incoming plasmid, not the resident, which was attacked.

A possible explanation is provided by the recent observation (M. E. Nugent, unpublished) that R40a is in fact two plasmids of approximately 60 and 85 Md. The larger of these is of a size typical of plasmids belonging to incompatibility groups A and C (Barth and Grinter, unpublished). The results can be explained if this is a non-transmissible IncC plasmid carrying the antibiotic resistance genes, and the other plasmid is conjugative, IncY, and has no antibiotic resistances. The cryptic plasmid is responsible for the transfer of the resistance plasmid by mobilisation.[1] Transfer of both plasmids is reduced when the recipient carries an IncY plasmid by exclusion of the mobilising plasmid. Where transfer is successful, the recipient has two incompatible IncY plasmids which in the absence of selection for either, segregate randomly, 50% of the recipients losing one plasmid and 50% losing the other. This would explain the "dislodgement" of P1Cm and Tc19 by R40a. Finally, in those "doubles" harbouring Tc19 and the IncC plasmid, the cryptic mobilising plasmid has been eliminated by incompatibility with Tc19, and so the IncC plasmid will not transfer. (It cannot be mobilised by Tc19 which, like P1Cm, transfers by transduction.) This explanation, although bizarre, does fit in with the observations and should be easy to test by looking at the DNA of the various plasmid combinations. This work has not been undertaken.

Regarding the incompatibility observed between two IncC plasmids (R40a and R57b-1) and two of IncA(Tc10, Tc11), it suggests the distinctions between the groups A and C may not be clear cut. Many plasmids are already designated A-C (Hedges, 1977). The status of IncA plasmids should be reassessed.

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1 Spontaneous loss of transmissibility of R40a has been observed (N. Grinter, unpublished).

Properties of Tc1. This plasmid was unusual in that its phenotype (TSu $Sex^{+/-}$ Tra^{-}) cannot be explained by a single deletion from the map shown in Figure 6.1. Two plasmids, Cm17 (CSu $Sex^{+/-}$ Tra^{-}) and Tc21 (TSu Sex^{-} $Tra^{defective}$), however, do suggest that the gene order is as shown on the map. Tc1 might be another P1 prophage like Tc19 and so its compatibility with P1Cm and RA1-1b was tested. Tc21 was also tested because it was the only plasmid apart from Tc19 with the $Tra^{defective}$ phenotype, all other transductants being either Tra^{-} or transferring with the same frequency as RA1-1.

Both plasmids were found to co-exist stably with P1Cm in all cases tested (12/12) and to be eliminated by RA1-1b (12/12), and so belong to IncA. Thus, Tc1 may have originated in a packaging event which introduced more than one deletion in the RA1-1 parent molecule. An alternative explanation is that there is a certain amount of overlap between the Sex and Tra regions of RA1-1 as has been shown with RP4 where insertional mutations in the Sex region also gave a Tra^{-} phenotype (Barth, manuscript in preparation).

Molecular characterisation of the transductants: experimental rationale. The relationship between RA1-1 and its transductants was studied by comparing the molecular fragments produced by cleavage of their DNAs with a restriction endonuclease. Such an enzyme recognises a specific base sequence on the DNA molecule and produces a staggered, double-stranded cut in this sequence. For the enzyme used here, *Bgl*II, this sequence (5' → 3') is $\overset{\downarrow}{A}GATCT$ / $TCTAG\overset{\uparrow}{A}$ with the cut sites shown by the arrows (Roberts, 1977). Assuming that continuous sequences of DNA are packed into transducing particles (Lee *et al.*, 1974), then a study of fragment patterns of the various transductants will show:

1. Fragments which do not carry essential plasmid functions can be lost. All fragments either lost or altered in a particular trans-

ductant were originally a continuous section of the parental plasmid.

2. Fragments which are always retained by the transductants will carry essential plasmid functions or be from regions on the parent plasmid which lie between essential genes and the selected drug-resistance marker.

3. Fragments lost or altered in all the members of a particular phenotypic class of transductants contain the functions missing from this class.

4. If packaging of host DNA into phage particles is initiated from specific sites on that DNA, then the number of different transducing particles which can be produced from a plasmid is limited to the number of such sites on the plasmid molecule. If host DNA is packaged at random, however, then a whole spectrum of transducing particles can be produced. Comparison of the digest patterns of the transductants, particularly those having the same phenotype, may provide evidence favouring one of these models. [An additional source of variation between the transductants could be introduced by the process leading to the recircularising of the transduced plasmid in its new host. This has been shown to be *recA* independent (see above). Two possible mechanisms for recircularisation are recombination between insertion sequences on the plasmid, and ligation of sticky ends produced on the transduced DNA by a restriction-type endonuclease involved in packaging DNA into phage heads. The former of these is thought to be unlikely, for if such an illegitimate event were possible it would also be seen in the parent plasmid which would therefore tend to produce spontaneous deletion mutants. These have not been observed. If the second mechanism were involved, then as suggested above only a limited number of types of transductant would be produced. (Also, transduction of plasmid DNA would not be possible

into a ligase-deficient mutant.)]

Molecular characterisation of the transductants: experimental results. Plasmid DNA was prepared from 24 of the transductants described in Table 6.1 by caesium chloride/ethidium bromide density gradient ultracentrifugation. These included five representatives from each of the four major, well-defined phenotypic groups (numbers 2-5, Table 6.1(b)) as well as some of the minor classes. Each purified DNA was digested with restriction endonuclease *Bgl*II and subjected to electrophoresis in a horizontal agarose gel as described in Chapter 1. λ DNA cut with *Bgl*II, *Eco*R1, or *Bgl*II and *Eco*R1 was used as a standard. The gels were run for about 3 hours, by which time the smallest λ fragment was near the bottom. They were removed, photographed, replaced in the gel holder, sealed with fresh agarose and run for a further 2-3 hours. This gave better resolution of the larger DNA fragments. The results are shown in Plates 6.1-6.3.

Molecular weights of the fragments were read from calibration curves constructed from the mobilities of the λ DNA fragments (Figure 6.5) and the results are shown in Figures 6.2-6.4.

Molecular weights of the intact plasmids were estimated from single colony gels (Chapter 1). RP4, R1 and RA1-1 were used as molecular weight standards. The gels are shown in Plates 6.4-6.7 and the calculated molecular weights in Table 6.4. A calibration curve is shown in Figure 6.6.

Tc19 was also compared with P1 prophage DNA and with P1Cm. *Bgl*II digests of all three plasmids were subjected to electrophoresis and the results shown in Plate 6.8. Molecular weights of the fragments were extrapolated from a calibration curve constructed from the mobilities of the P1 *Bgl*II fragments and their published molecular weights (Bächi and Arber, 1977). The results are shown in Figure 6.7.

Legend to Figures 6.2-6.4: Molecular weights of the BglII restriction fragments of RA1-1 and its transductants.

Molecular weights were extrapolated from a calibration curve based on the mobilities of λ DNA fragments (Figure 6.5) and the results drawn on a logarithmic scale. The weights assigned to the fragments of RA1-1 are the mean of three separate determinations. Each fragment is shown as a horizontal bar, with thicker bars denoting unresolved fragments. The number of unresolved fragments in a band is shown as the suffix x 2 or x 3 after the molecular weight.* Where bands present in RA1-1 are missing from the transductants they are shown as dotted lines. New bands appearing in the transductants are shown with their molecular weights written in. Changes in the number of fragments in a band are also recorded. Fragments shown in brackets are probably products of incomplete digestion, and have not been included in the calculation of total molecular weight. Where DNA concentrations were insufficient to allow the presence or absence of a band to be decided, a question mark is shown. At the foot of each column is the total molecular weight of the fragments with the molecular weight of the intact plasmid (determined from single colony gels, see Plates 6.4-6.7 and Table 6.4) shown beneath in brackets.

* The number of fragments in a band is decided from the relative intensities of the bands. In a series of single fragments of decreasing molecular weight, there is a gradual decrease in brightness due to there being less DNA in each successively lighter band. Any band which does not fit into this sequence contains either more or less fragments than its neighbours.

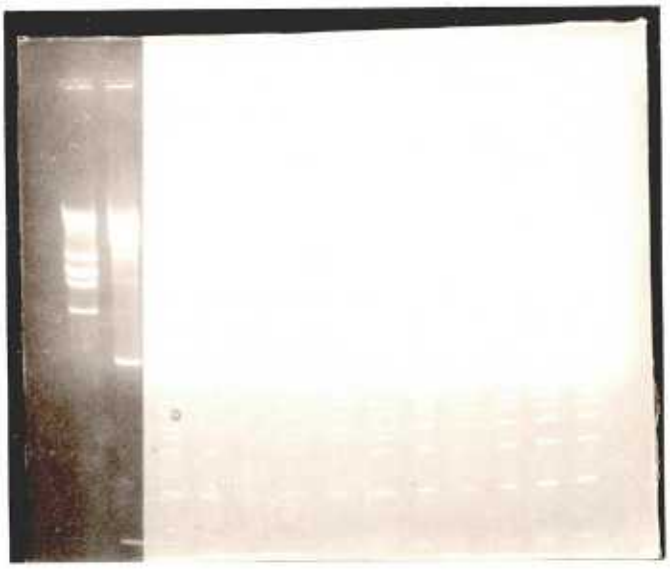
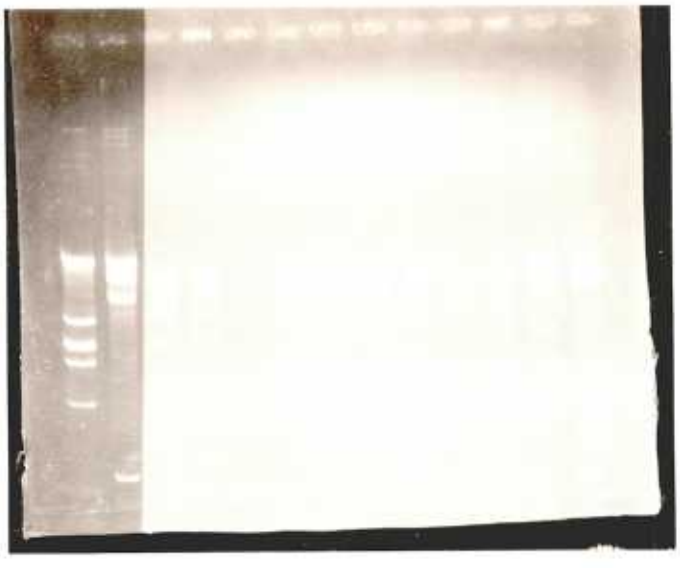


Plate 6.1. BglII digests of RA1-1 and its transductants.

Plasmid DNA after cleavage with restriction endonuclease and electrophoresis in 0.7% agarose/borate (Chapter 1). The upper photograph shows the migration after 3 hours and the lower after a further 2 hours. The tracks are: 1, λ EcoR1; 2, λ BglII; 3, RA1-1; 4, Cm1; 5, Cm6; 6, Cm24; 7, Tc3; 8, Tc20; 9, Cm3; 10, Cm9; 11, Cm15; 12, Cm22; 13, Tc17. Plasmids in tracks 4-8 are phenotypically indistinguishable from the parent (i.e. $C^{R}T^{R}Su^{R}Sex^{+}Tra^{+}$), and those in tracks 9-13 are $C^{R}T^{R}Su^{R}Sex^{+}Tra^{-}$. The bands in tracks 1 and 2 were more intense than the others and so were given less exposure. The molecular weights of the fragments are shown in Figure 6.2.



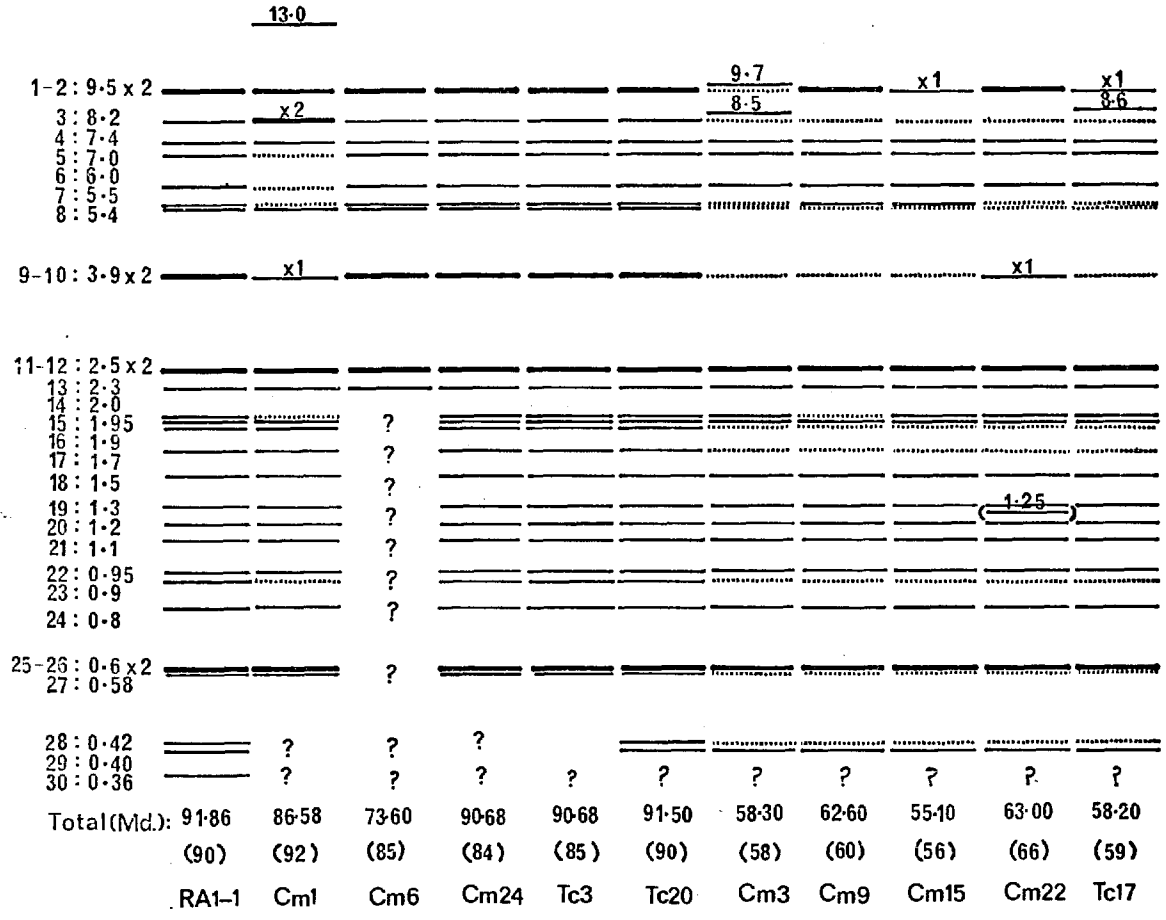


Figure 6.2 : BglII fragments of RA1-1 and its transductants (from Plate 6.1) .

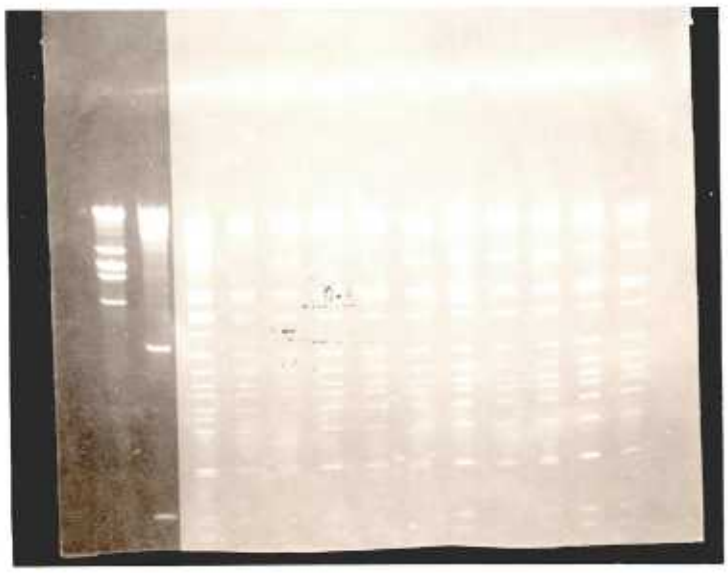
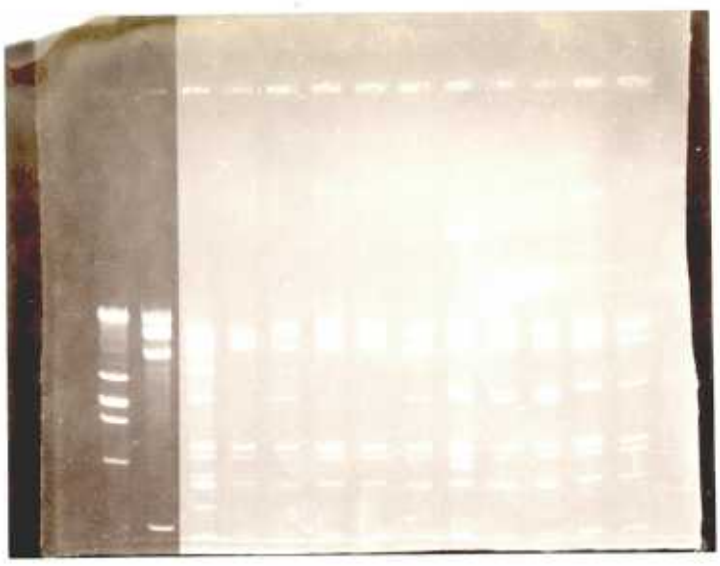


Plate 6.2. BglII digests of RA1-1 and its transductants.

Plasmid DNA after cleavage with restriction endonuclease and electrophoresis in 0.7% agarose/borate (Chapter 1). The upper photograph shows the migration after 3 hours and the lower after a further $2\frac{1}{4}$ hours. The tracks are: 1, λ EcoR1; 2, λ BglII; 3, RA1-1; 4, Tc2; 5, Tc8; 6, Tc13; 7, Tc18; 8, Tc24; 9, Tc4; 10, Tc6; 11, Tc9; 12, Tc11; 13, Tc15. Plasmids in tracks 4-8 are phenotypically $C^{R}T^{R}Su^{R}Sex^{-}Tra^{-}$, and those in tracks 9-13 are $C^{S}T^{R}Su^{R}Sex^{-}Tra^{-}$. The bands in tracks 1 and 2 were more intense than the others and so were given less exposure in the upper photograph. The molecular weights of the fragments are shown in figure 6.3.



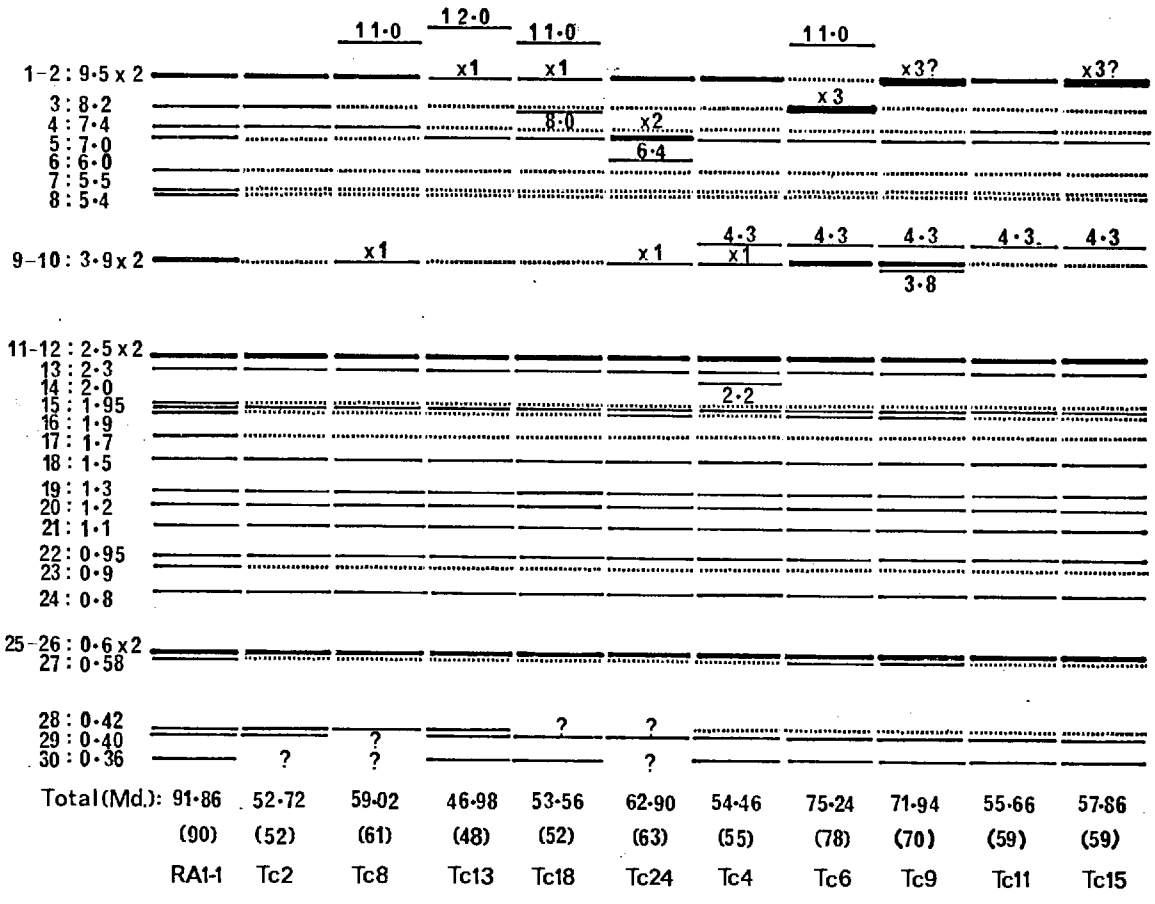


Figure 6.3 : BglII fragments of RA1-1 and its transductants (from Plate 6.2) :

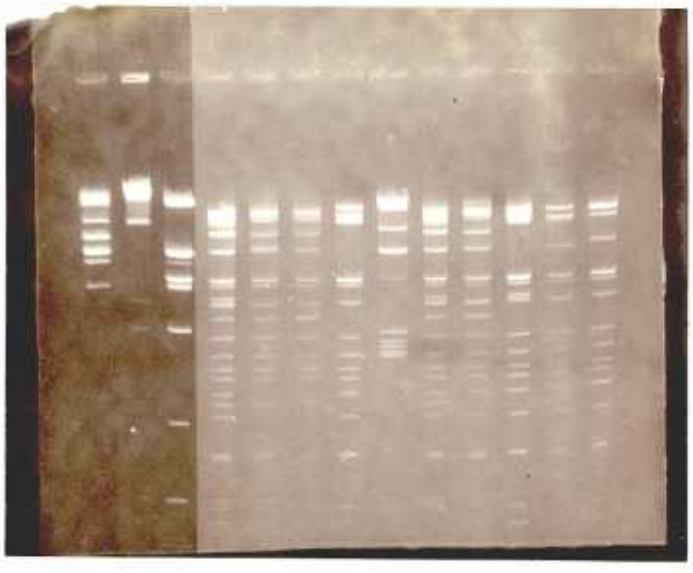
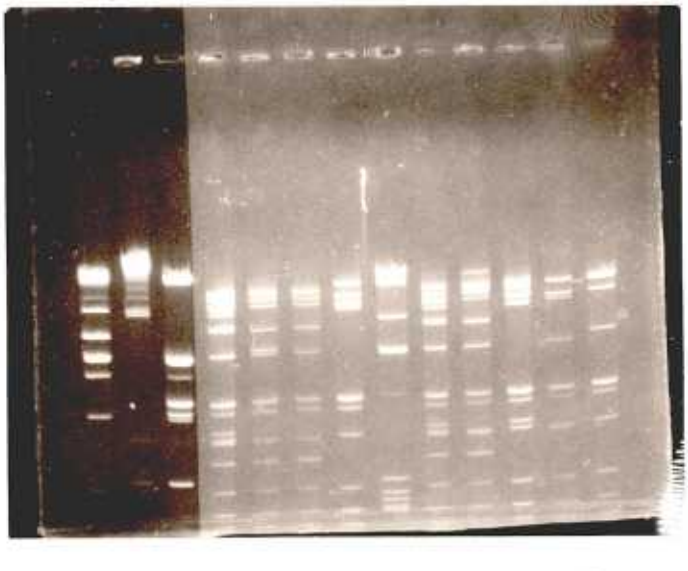


Plate 6.3. BglII digests of RA1-1 and its transductants.

Plasmid DNA after cleavage with restriction endonuclease and electrophoresis in 0.7% agarose/borate (Chapter 1). The upper photograph shows the migration after 3 hours and the lower after a further 2 hours. The tracks are (with phenotype where appropriate): 1, λ EcoR1; 2, λ BglII; 3, λ BglII + EcoRI; 4, RA1-1; 5 and 6, Tc10 and Tc22 ($C^{S}T^{R}Su^{R}Sex^{+}Tra^{+}$); 7, Cml7($C^{R}T^{S}Sex^{+/-}Tra^{-}$); 8, Tc19($C^{S}T^{R}Su^{S}Sex^{+}Tra^{def}$); 9 and 10, Tc3 and Cml($C^{R}T^{R}Su^{R}Sex^{+}Tra^{+}$); 11, Tc17;($C^{R}T^{R}Su^{R}Sex^{+}Tra^{-}$); 12, Tc24($C^{R}T^{R}Su^{R}Sex^{-}Tra^{-}$); 13, Tc15($C^{S}T^{R}Su^{R}Sex^{-}Tra^{-}$). The bands in tracks 1-3 were more intense than the others and so were given less exposure. λ DNA in these tracks has given extra bands due to incomplete digestion (except in track 2 which has hardly digested at all). The products of complete digestion were located by comparisons with other (complete) λ digests and were then used to calibrate the gel. The molecular weights of the fragments are shown in Figure 6.4.



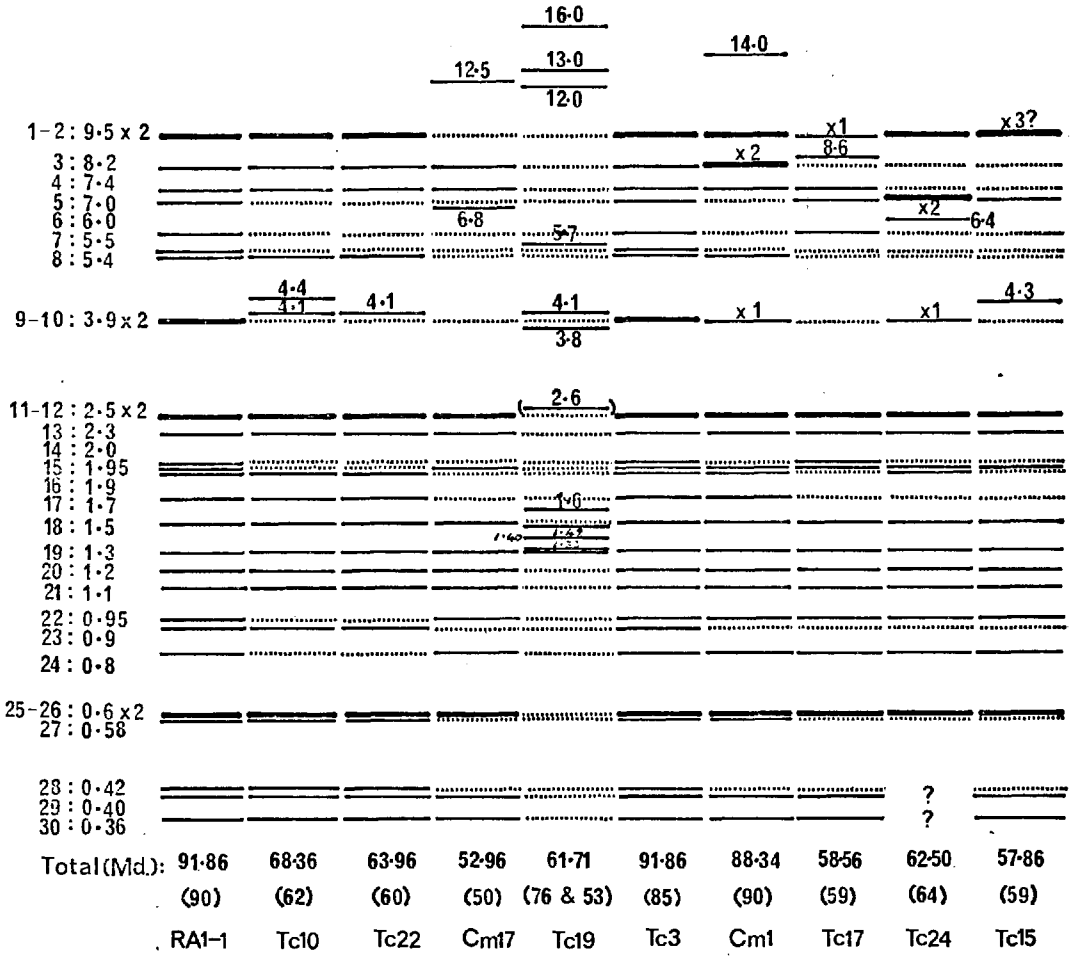


Figure 6.4 : BglII fragments of RA1-1 and its transductants (from Plate 6.3).

Figure 6.5. Calibration curve for λ DNA.

The points represent the migration of λ DNA fragments after electrophoresis in 0.8% agarose/borate for 3 and 5 hours. The circles show the migration of fragments from EcoR1 digestion, and the squares those from BglII cutting. Fragment sizes are from Thomas and Davis (1975) and Kamp et al. (1977). Molecular weight of λ was taken as 32.37 (Murray, unpublished).

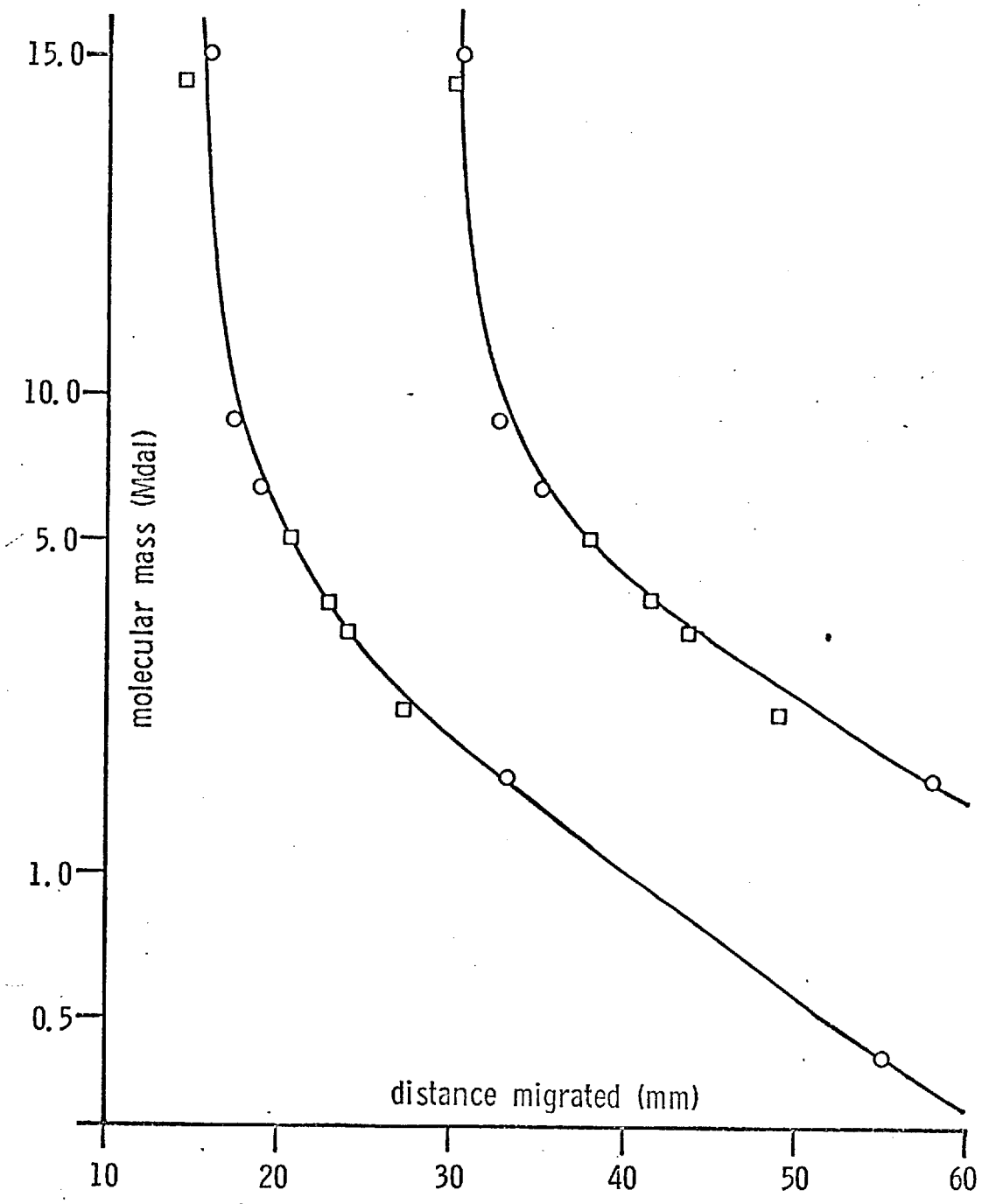


Figure 6.5 : Calibration curve for λ DNA .

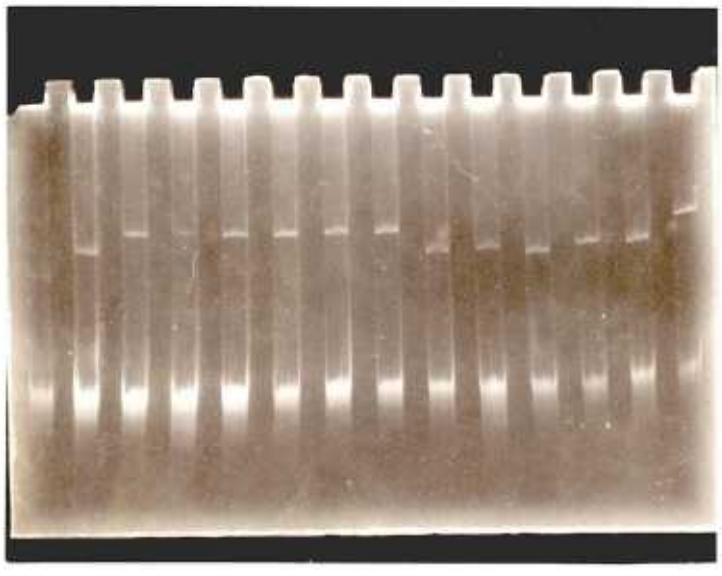


Plate 6.4. Single colony gel of RA1-1 transductants

Single colonies of strains containing RA1-1 or its transductants were lysed on top of the 0.8% agarose and subjected to borate gel electrophoresis as described in Chapter 1. The tracks are: 1, RP4; 2, R1; 3, RA1-1; 4, Cm1; 5, Cm6; 6, Cm24; 7, Tc3; 8, Tc20; 9, Cm3; 10, Cm9; 11, Cm15; 12, Cm22; 13, Tc17; 14, RA1-1. Plasmids in tracks 4-8 are phenotypically indistinguishable from RA1-1 ($C^{R}T^{R}Su^{R}$ $Sex^{+}Tra^{+}$). Those in tracks 9-13 are phenotypically $C^{R}T^{R}Su^{R}Sex^{+}Tra^{-}$. The upper bands in each track are plasmid DNA and the lower, more diffuse bands are chromosome. Judging by the position of the chromosome in each track, it looks as if tracks 13 and 14 have run more slowly than the rest and consequently molecular weight estimates from these tracks would be unreliable. Tc17 was re-run and the result shown in Plate 6.7. Calculated molecular weights are shown in Table 6.4.

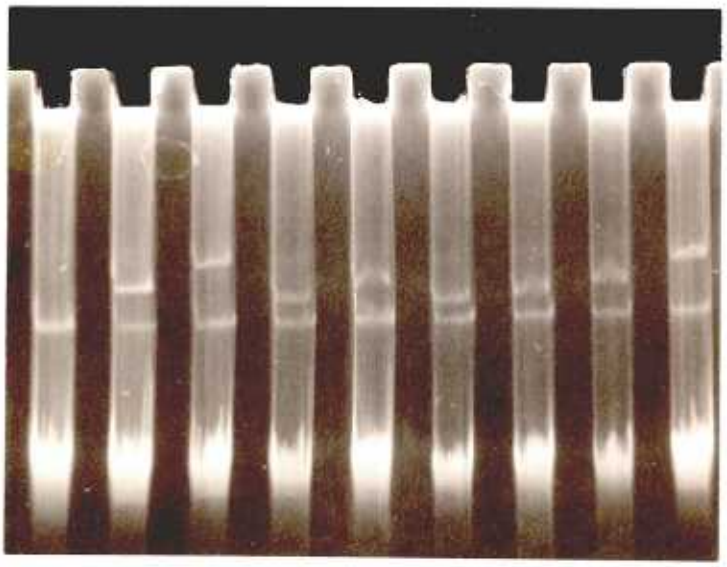


Plate 6.5. Single colony gels of RA1-1 transductants

Single colonies of strains containing RA1-1 or its transductants were lysed on top of a 0.8% agarose/borate gel and subjected to electrophoresis as described in Chapter 1. The tracks are: 1, RP4; 2, R1; 3, RA1-1; 4, Tc2; 5, Tc8; 6, Tc13; 7, Tc18; 8, Tc24; 9, RA1-1. The transductants are phenotypically $C^{R}T^{R}Su^{R}Sex^{-}Tra^{-}$. The bright, diffuse band near the bottom is chromosome. To improve the calibration of the gel, RP4 was included in each track as an internal standard. For each plasmid, the distance run was multiplied by the ratio of the distance run by RP4 in track 1 to the distance run by the RP4 internal standard, and this figure used to estimate the molecular weight from the calibration curve. This method ensured that minor irregularities in the running of the gel would not interfere with the results. However, the plasmid bands obtained were less sharp (possibly through overloading the gel) and consequently more difficult to measure, and so the method was not repeated. Calculated molecular weights are shown in Table 6.4.

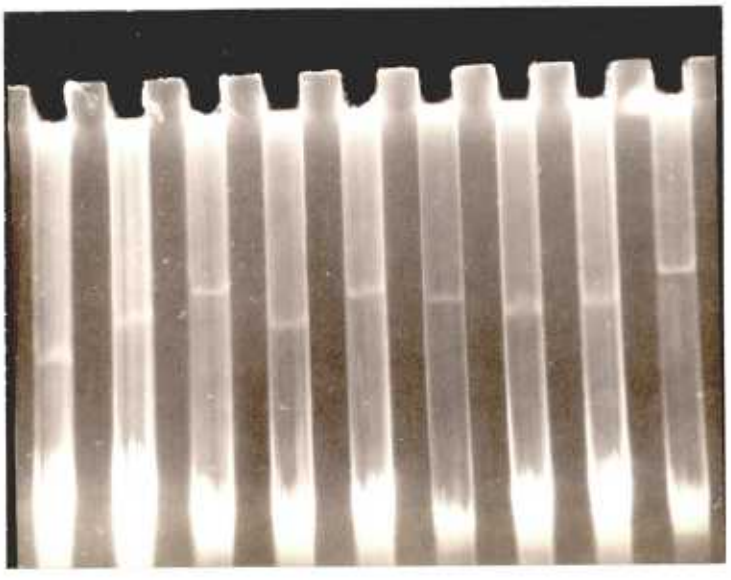


Plate 6.6. Single colony gel of RA1-1 transductants.

Single colonies of strains containing RA1-1 or its transductants were lysed on top of the 0.8% agarose/borate gel and subjected to electrophoresis as described in Chapter 1. The tracks are: 1, RP4; 2, R1; 3, RA1-1; 4, Tc4; 5, Tc6; 6, Tc9; 7, Tc11; 8, Tc15; 9, RA1-1. Plasmids in tracks 4-8 are phenotypically $C^{S}T^{R}Su^{R}Sex^{-}Tra^{-}$. The upper bands are plasmid and the lower, brighter and more diffuse bands are chromosome. Calculated molecular weights are shown in Table 6.4.

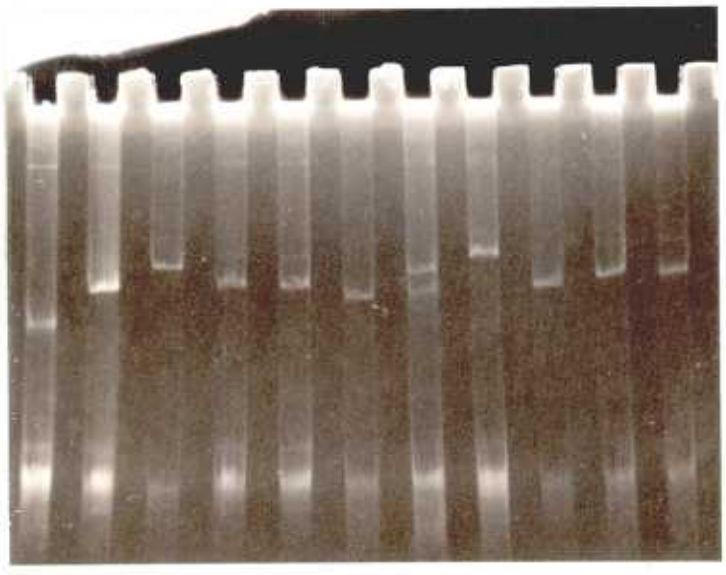


Plate 6.7. Single colony gel of RA1-1 transductants

Single colonies of strains containing RA1-1 or its transductants were lysed on top of a 0.8% agarose/borate gel and subjected to electrophoresis as described in Chapter 1. The tracks (and phenotypes) are: 1, RP4; 2, R1; 3, RA1-1 ($C^{R}T^{R}Su^{R}Sex^{+}Tra^{+}$); 4 and 5, Tc10 and Tc22 ($C^{S}T^{R}Su^{R}Sex^{+}Tra^{+}$); 6, Cm17 ($C^{R}T^{S}Su^{R}Sex^{+/-}Tra^{-}$); 7, Tc19 ($C^{S}T^{R}Su^{S}Sex^{+}Tra^{def.}$); 8, Cm1 ($C^{R}T^{R}Su^{R}Sex^{+}Tra^{+}$); 9, Tc17 ($C^{R}T^{R}Su^{R}Sex^{+}Tra^{-}$); 10, Tc24 ($C^{R}T^{R}Su^{R}Sex^{-}Tra^{-}$); 11, Tc15 ($C^{S}T^{R}Su^{R}Sex^{-}Tra^{-}$). The broad band near the bottom is chromosome. There are faint bands just above the chromosome and just below the origin in some tracks. These are considered irrelevant because they can be seen in the standards as well. There are also faint bands about 0.5 cm above the plasmid bands in tracks 4, 5, 10 and 11 which are considered to be artifacts because: i) they would be heavier than RA1-1 if genuine; ii) where the same plasmids were retested, no evidence for a second band was seen [see Plate 6.5, track 8 (Tc24) and Plate 6.6, track 8 (Tc15)]. Calculated molecular weights are shown in Table 6.4.

Figure 6.6. Calibration curve for single colony gels.

Molecular weights of the standards are: RA1-1, 90 Md. (Barth, unpublished); R1, 62 Md. (Jacob et al., 1977a); RP4, 36 Md. (Barth and Grinter, 1977a).

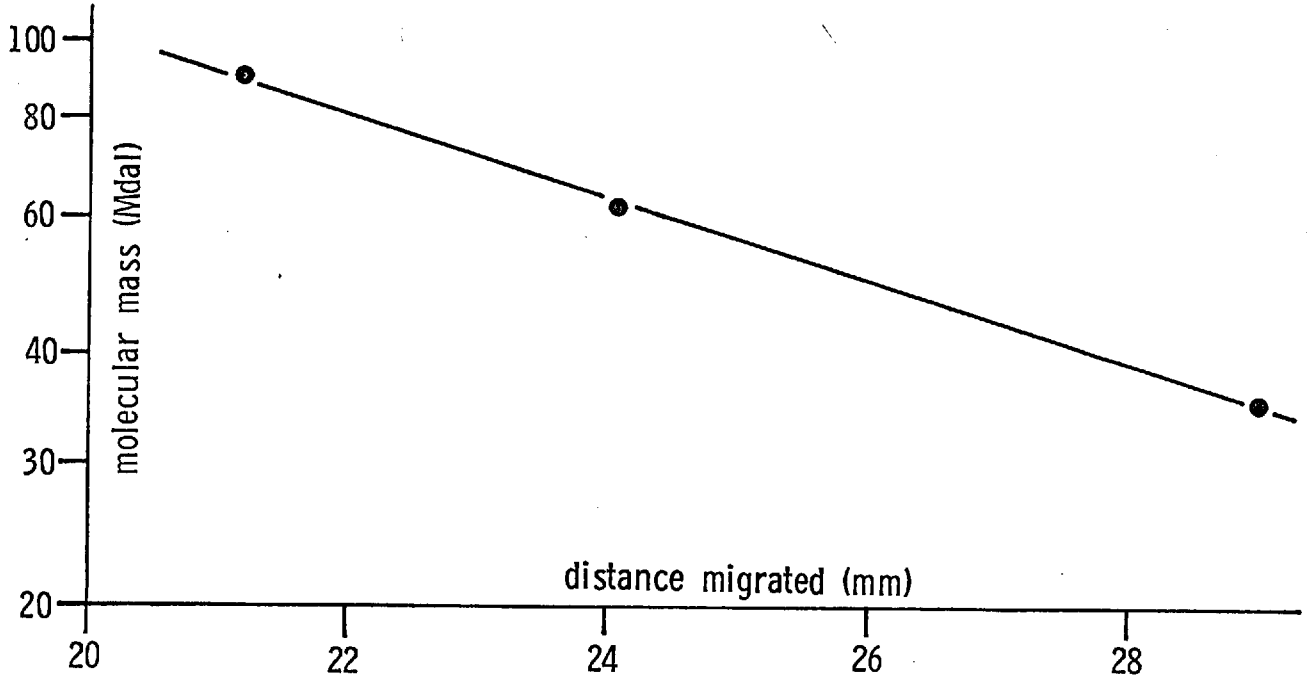


Figure 6.6 : Calibration curve for single colony gels.

Table 6.4. Molecular weights of RA1-1 transductants

Plasmid	Phenotype	Molecular weight in Megadaltons (to nearest whole number)	
		sc[1]	rf[1]
RA1-1	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{+}	90	92
Cm1	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{+}	92[2]	87
Cm6	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{+}	85	> 74
Cm24	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{+}	84	91
Tc3	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{+}	85	91
Tc20	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{+}	90	92
Cm3	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{-}	58	58
Cm9	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{-}	60	63
Cm15	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{-}	56	55
Cm22	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{-}	66	63
Tc17	$C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{-}	59	58
Tc2	$C^{R}T^{R}Su^{R}$ Sex^{-} Tra^{-}	52	53
Tc8	$C^{R}T^{R}Su^{R}$ Sex^{-} Tra^{-}	61	59
Tc13	$C^{R}T^{R}Su^{R}$ Sex^{-} Tra^{-}	48	47
Tc18	$C^{R}T^{R}Su^{R}$ Sex^{-} Tra^{-}	52	54
Tc24	$C^{R}T^{R}Su^{R}$ Sex^{-} Tra^{-}	63[2]	63
Tc4	$C^{S}T^{R}Su^{R}$ Sex^{-} Tra^{-}	55	54
Tc6	$C^{S}T^{R}Su^{R}$ Sex^{-} Tra^{-}	78	75
Tc9	$C^{S}T^{R}Su^{R}$ Sex^{-} Tra^{-}	70	72
Tc11	$C^{S}T^{R}Su^{R}$ Sex^{-} Tra^{-}	59	56
Tc15	$C^{S}T^{R}Su^{R}$ Sex^{-} Tra^{-}	62[2]	58
Tc10	$C^{S}T^{R}Su^{R}$ Sex^{+} Tra^{+}	62	68
Tc22	$C^{S}T^{R}Su^{R}$ Sex^{+} Tra^{+}	60	64
Cm17	$C^{R}T^{S}Su^{R}$ $Sex^{+/-}$ Tra^{-}	50	53
Tc19	$C^{S}T^{R}Su^{S}$ Sex^{+} Tra^{def}	76 + 53[3]	62

Footnotes to Table 6.4.

1 sc - derived from single colony lysates.
rf - derived from summation of restriction fragments.

2 Mean of two determinations.

3 Several attempts at single colony lysis of Tc19 failed to give a strong plasmid band. Two bands were seen in the gel shown in Plate 6.7 and it is these that appear in the table.



Plate 6.8. BglII digests of P1 prophage DNA and related plasmids.

Plasmid DNA after cleavage and electrophoresis in 0.8% agarose/borate (Chapter 1) for 4 hours. The tracks are: 1, P1; 2, P1Cm; 3, Tc19. The molecular weights of the fragments are shown in Figure 6.7.

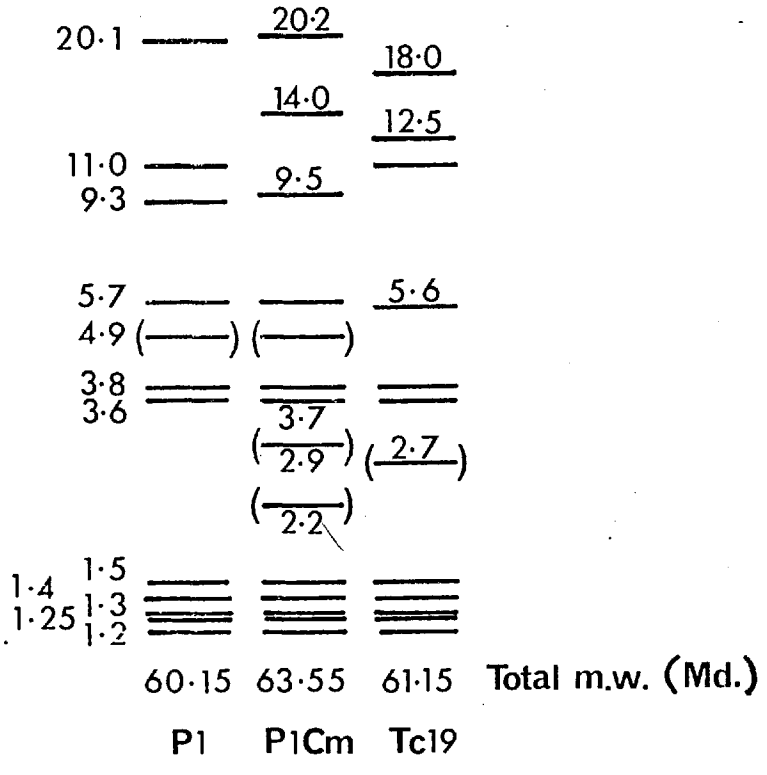


Figure 6.7: BglII fragments of P1 and derived specialised transducing phages (from plate 6.8).

Fragments are drawn as horizontal bars on a logarithmic scale. molecular weights of the BglII fragments of P1 are from Bächi and Arber, 1977. Bands differing in size from those produced by P1 have their molecular weights written in. Products of partial digestion (see legend Figure 6.2) are shown in brackets and are not included in the total molecular weight.

1. Analysis of plasmids phenotypically indistinguishable from the RA1-1 parent [Phenotypic class 5 - Table 6.1(b)]

*Bgl*II digestion of RA1-1 gave 30 fragments of 0.36 Md and above. The mean of three separate determinations of their molecular weight is shown in Figures 6.2-6.4. Total molecular weight of the fragments was 92 Md. Cm24, Tc3 and Tc20 (Figure 6.2) give a restriction digest pattern indistinguishable from that of RA1-1. Cm6 DNA was insuffiently concentrated to see fragments below 2 Md., but the visible parts of the digest are identical with RA1-1. Only Cm1 shows marked differences from the control, with fragments 5, 6, 7, 9 or 10, 14 and 23 missing. In their stead, two new fragments are found at 13 Md and 8.2 Md. The overall change in molecular weight is about 5 Md. Single colony gels on the complete plasmids showed there was little difference between them and the RA1-1 parent. Detection of changes in molecular weight of a few Megadaltons in plasmids of this size represents the resolution limit of the single colony technique, and so the observed differences are probably not signficant.

These results show that P1 is capable of transducing plasmids of up to 90 Md. molecular weight.

2. Analysis of plasmids from phenotypic classes 2, 3 and 4 [Table 6.1(b)]

Table 6.5 shows the fragments present in RA1-1 which are missing or altered in each of phenotypic classes 2, 3 and 4. Only those fragments which are absent from all five members of each group are shown in the table. While there are differences between transductants from the same phenotypic class, the absence of a particular fragment from each member of a particular class suggests that it carries genes responsible for the functions missing from that class (or is adjacent to such a fragment). The table shows that fragments 8, 17 and 23 (total m.w. 8 Md.) may bear genes for plasmid transfer, and fragments 6, 7 and 14 (total m.w. 13.5 Md.)

may code for surface exclusion. These are probably not all the fragments with genes for these functions. If RA1-1 resembles F, it is unnecessary to delete all genes for transfer or surface exclusion to produce a Tra^- or Sex^- phenotype (Manning and Achtman, 1979).

The other fragments missing from class 2 (numbers 3, 9 or 10, 16, 27 and 28) are probably adjacent to the transfer genes (if the transductants contain a single deletion). Similarly, fragments 9 or 10, 27 and 28 are probably adjacent to the surface exclusion genes. Since some fragments (9 or 10, 27 and 28) are close to both sets of genes, then surface exclusion and transfer genes are close to each other on the RA1-1 plasmid. This is compatible with the map derived from transduction data shown in Figure 6.1.

It is not possible to assign drug resistance functions to particular restriction fragments which might be expected since the amount of DNA coding for them is probably small. However, the one tetracycline sensitive derivative isolated (Cm17, $C^R Su^R$ $Sex^{+/-} Tra^-$) has missing from it fragments 1, 2, 5, 9, 10, 16, 27 and 28 in addition to fragments 6, 7, 8, 14, 17 and 23 which it is believed are concerned with transfer and surface exclusion. Numbers 9 or 10, 16, 27 and 28 are also missing from some of the Tra^- or Sex^- Tra^- plasmids shown in Table 6.5. This shows the tetracycline resistance genes are close to the same Tra and Sex functions which are missing from phenotypic classes 2, 3 and 4, instead of being associated with other genes for these functions situated elsewhere on the plasmid. This fits with the genetic map shown in Figure 6.1.

Also, the similarity between the class 3 (CTSu Sex^-Tra^-) and class 4 (TSu Sex^-Tra^-) transductants (Table 6.5) suggests that chloramphenicol resistance is also close to the same Sex and Tra genes, as shown in Figure 6.1.

Table 6.5. *Bgl*II restriction fragments of RA1-1 not present in transductants

Phenotypic class[1]	Missing *Bgl*II fragments[2]
2 ($C^{R}T^{R}Su^{R}Sex^{+}Tra^{-}$)	3, 8, 9 or 10, 16, 17, 23, 27, 28
3 ($C^{R}T^{R}Su^{R}Sex^{-}Tra^{-}$)	6, 7, 8, 9 or 10, 14, 17, 23, 27
4 ($C^{S}T^{R}Su^{R}Sex^{-}Tra^{-}$)	6, 7, 8, 14, 17, 23, 28

Fragments missing from each of classes 2, 3 and 4 are shown surrounded by dotted lines, as are those fragments missing only from classes 3 and 4. Consideration of the phenotypes of the transductants suggests that fragments 8, 17 and 23 carry genes involved in plasmid transfer, and fragments 6, 7 and 14 carry genes for surface exclusion.

1 Phenotypic classes are as shown in Table 6.1 (b).

2 The fragment numbers are those given in Figure 6.2 to the *Bgl*II digest of RA1-1. Only those fragments missing from all five members of each group are shown in the table.

3. Analysis of phenotypic classes 6, 7 and 10

Too few representatives of these classes were isolated to draw the sort of conclusions presented above. However, the close resemblance between the two examples of class 6 (Tc10 and Tc22, TSu $Sex^{+}Tra^{+}$) - they differ only in Tc10 having an additional 4.4 Md fragment which is not present in Tc22 (Figure 6.4) - suggests that there is very little latitude for separating the chloramphenicol resistance genes from Sex and Tra functions, as was inferred in the previous section. CM17 (class 7, CSu $Sex^{+/-}Tra^{-}$) illustrates that the tetracycline resistance gene(s) are also close to Sex/Tra (see section 2).

The single representative of class 10, Tc19 (Figure 6.4, Plate 6.3), is clearly unrelated to RA1-1. Incompatibility tests showed it to be an IncY plasmid (see above) and accordingly its digest pattern was compared with two other IncY plasmids, P1 and P1Cm. The results are shown in Plate 6.8 and Figure 6.7. Ignoring small changes in fragment sizes (< 3%, which may be within experimental error) compared to those produced from P1, the digest of P1Cm is most simply interpreted as resulting from a 3 Md insertion into the 11 Md fragment of P1. The size and site of the insertion is in general agreement with other published results (Yun and Vapnek, 1977; Yarmolinsky, 1977).

The digest of Tc19 shows it probably has nine fragments in common with P1. The remaining two fragments are 12.5 and 18 Md compared with 9.3 and 20.1 Md for the remaining two from P1. This is consistent with an insertion of about 3 Md in the 9.3 Md P1 fragment accompanied by a 2 Md deletion from the 20.1 Md piece. Insertions have been reported in the 9.3 Md *Bgl*II fragment of P1 (Yarmolinsky, 1977). P1 packaging is believed to start preferentially on the 20.1 Md fragment (Bächi and Arber, 1977) and so deletions of the phage genome as the result of a large insertion would also preferentially start in or near the

same fragment (assuming packaging is of concatamers by a headful mechanism). It is possible, however, that the insertion is of a larger piece (bearing its own *Bgl*II sites(s)) into the 5.7 Md. fragment with accompanying changes in the 9.3 and 20.1 Md. fragments which lie on either side of it in the P1 genome (Bächi and Arber, 1977).

Tc19, apart from its prophage nature, was unique among the transductants in that the estimate of its molecular weight from summing the *Bgl*II restriction fragments was unlike that obtained from single colony gels on the intact plasmid (Table 6.4). Indeed, the latter showed the presence of two plasmids, neither of which had a molecular weight near to that of c. 60 Md. derived from two separate summations of restriction fragments (Figures 6.4 and 6.7). It proved very difficult to obtain clear plasmid bands from Tc19 by the single colony lysis technique. Generally, only very little material was found in the region of the gel where plasmid DNA would be expected, and this tended to be smeared rather than organised into bands. Thus the result shown in Plate 6.7 may be an artifact. The apparent breakdown of plasmid DNA usually seen when this technique is applied to Tc19 may be a characteristic of the P1 prophage and is possibly the result of a plasmid-specified nuclease. Comparison with other IncY plasmids will test this interpretation, but this has not been attempted here.

4. Location of essential and non-essential regions on RA1-1

Comparisons between transductants, particularly the smaller ones, show that the following *Bgl*II fragments of RA1-1 can be wholly or partly deleted: 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 14, 15, 16, 17, 22, 23, 24, 27, 28. The presence of such large amounts of apparently non-essential DNA on RA1-1 may be because it was studied in an *E. coli* host, and not *Aeromonas liquefaciens* in which RA1 was originally isolated. RA1 may be highly adapted to its natural host and carry

large numbers of genes specifically concerned with plasmid maintenance in that host. There may also be environmental challenges to that host which the plasmid equips it to meet but which are not present in the laboratory.

Fragments 11, 12, 13, 18, 19, 20, 21, 25 and 26 (total molecular weight 13.6 Md.) are present in every transductant (except Tc19, of course). These fragments presumably carry essential (maintenance) genes and the material between such genes and the selected drug resistance genes.

5. Mechanism of packaging of plasmid DNA into transducing particles

Within a phenotypic class of transductants there is a certain amount of heterogeneity. Considering the amount of apparently non-essential DNA on RA1-1 (section 4), however, the heterogeneity is perhaps less than would be expected if plasmid DNA were packaged at random. More concrete evidence comes from consideration of phenotypic class 6 ($T^R Su^R$ Sex^+ Tra^+, Tc10 and Tc22, Figure 6.4). These two plasmids give identical digest patterns with the exception of an extra 4.4 Md. fragment found in Tc10. The only other fragment generated from these plasmids which is not found in the RA1-1 digest is a 4.1 Md. piece which both transductants have. It is conceivable that both plasmids were generated by packaging being initiated at a specific site on the plasmid genome (giving rise to the 4.1 Md. digest fragment by shortening one of the larger fragments). However, as pointed out in section 3, the close resemblance between Tc10 and Tc22 may be because the deletion producing them has to terminate between the genes for chloramphenicol resistance and surface exclusion/transfer which may be very close together.

By far the best evidence for packaging being initiated at particular sites on RA1-1 comes from the digests of plasmids in phenotypic class 4

($T^R Su^R$ Sex^- Tra^-, Figure 6.3). This group is the most heterogeneous with respect to molecular weight (mean 64.2, standard deviation 9.5) but all plasmids in the group generate a 4.3 Md. fragment after *BglII* cutting which is not present in RA1-1 digests. This fragment is probably created by a cut at a specific site on RA1-1 by a P1-coded packaging enzyme. The variation in plasmid size may be introduced at the packaging stage or when the plasmid recircularises.

There is also evidence for packaging not being of continuous lengths of DNA. For example, the transductant Tc1 (see above) confers a phenotype consistent with its having undergone either rearrangement or deletion of more than one region. [P1 transduction of chromosomal markers borne by F'14 has been shown to produce novel linkages in the recipient (Hendrickson and Duggan, 1976)]. If transductional shortening of a circular plasmid molecule is by the introduction of a single deletion (Lee *et al.*, 1974), this can generate only one novel restriction fragment in digests of the transductant. Several transductants, however, have at least two such fragments (Figures 6.2-6.4) and some may have three or four. This proves that multiple deletions and/or rearrangements can be generated when plasmid DNA is transduced by P1.

Summary of results

1. Examination of 48 P1 transductants of RA1-1 showed them to fall into ten different classes on the basis of their conferred phenotypes.

2. Consideration of the markers which were co-transducible (and hence those which were co-deletable) allowed a genetic map of RA1-1 to be deduced (Figure 6.1).

3. The *recA* gene product was not necessary for the reformation of circular plasmid molecules in the transductional recipient.

4. One transductant (Tc19) was shown to be a P1 prophage.

5. Molecular studies showed five transductants to be over 80 Md. molecular weight. Of these, at least three were indistinguishable from RA1-1, and one clearly distinguishable.

6. *Bgl*II restriction fragments of RA1-1 carrying genes for transfer, surface exclusion and plasmid maintenance were detected and certain aspects of the genetic map confirmed.

7. Large parts of RA1-1 carry no essential plasmid functions.

8. There was evidence for a site specific nuclease involved in packaging transducing particles.

9. P1 transduction of plasmid DNA can introduce multiple deletions and/or rearrangements.

Discussion

A. Transduction of oversized plasmids

P1 transduction of plasmid DNA of more than 60 Md. has been reported previously (Pittard and Adelberg, 1963, reported in Low, 1972; Sharp *et al.*, 1972; Rosner, 1975), although the authors give no indication of how common an event this is nor do they discuss the possible mechanisms involved. Hendrickson and Duggan, 1976, observed the transuction of most if not all of F'14 (c. 200 Md.), but their conclusions are based on genetic evidence rather than direct measurement of the transduced DNA.

Of the conceivable mechanisms for the observed transduction of the whole of RA1-1 (90 Md.), several are considered below.

1. Large P1 particles. The selections imposed in the transductions were for the essential genes of RA1-1 together with those conferring either tetracycline or chloramphenicol resistance. Since these can be present on a plasmid of less than 60 Md. (and are therefore transducible by a normal sized P1 particle), the only way that the products of large-headed transducing particles would be detected is if they formed a significant part of the population i.e. there is not selection for such transductants.

The calculations in the appendix to this chapter suggest that these hypothetical oversized particles would have been detected, possibly by direct observation (Walker and Anderson, 1970), and certainly by their purification in a caesium chloride gradient. Rosner, 1975, and Hendrickson and Duggan, 1976, searched for such particles without success. Either these particles do not exist or they do not form a band in a caesium chloride density gradient (i.e. they are not of uniform density). This latter possibility is supported by the observation that the ability of a P1 preparation to

transduce large DNA molecules is destroyed after purification in a caesium chloride gradient (Hendrickson and Duggan, 1976).

If transducing particles can contain widely varying amounts of host DNA, then where their DNA content has been measured, the results would represent an average. If the molecular weights of the RA1-1 transductants are averaged (taking into account the frequency with which each type occurs) the result is 63.4 Md. This is very little different from the figure of 60 derived by Ikeda and Tomizawa, 1965a, and may be an overestimate. The smallest amounts of transduced plasmid DNA are not detected because they cannot form replicons in the new host.

Large P1 particles would also presumably be able to transduce markers on the chromosome further apart than the 2.2 minutes which seems to be the maximum (Caro and Berg, 1971). Generally, there is good agreement between co-transduction frequencies and data from interrupted matings (Bachman *et al.*, 1976). It is possible that a mutation in P1 could alter the size and proportion of head size variants in the population, although such mutants have not been identified (Yarmolinsky, 1977). Alternatively, there may be special factors affecting packaging of plasmid DNA. This is considered further in section C.

Heteroduplex analysis (Lee *et al.*, 1974) of transduced F' DNA showed the plasmids in the recipient were all more or less the same size as the P1 prophage. This does not necessarily rule out the possibility that widely different amounts of DNA can be packaged. Their results could be due to constraints imposed by the selection on the size of plasmid which could be produced. An example of this is seen with the transduced RA1-1 plasmids belonging to phenotypic class 3 ($C^{R}T^{R}Su^{R}$ Sex^{+} Tra^{-}, Table 6.1(b)). 9/10 plasmids with this phenotype were selected on chloramphenicol and the mean and standard deviation of the molecular weight of the five of these examined is 59.8 ± 3.8.

2. Modified packaging. P1 phage heads may not normally be filled with DNA. When host DNA is being incorporated into transducing particles controls over DNA content may not be effective, allowing more DNA to be packaged on occasions. Against this is the finding that phage particles do not take up negative stain (Walker and Anderson, 1970), suggesting there is no unoccupied space within the heads. Alternatively, structural features of some plasmids may allow them to be packaged more efficiently and transduced in their entirety even though they are oversized (see Section C).

3. Duplication of plasmid sequences. Large plasmids could be produced in the transductional recipient by the transduced DNA undergoing duplication of certain regions. This would show up in restriction digests of oversized transductants where certain fragments would be present in more copies than in the parent plasmid, while others would be missing altogether. This is certainly not the case with three transductants (Tc20, Cm24 and Tc3, Figure 6.2) which appear to be identical with RA1-1.

4. Double or multiple transduction. Simultaneous transduction of a cell by two or more transducing particles carrying segments of RA1-1 could generate large plasmids in the recipient by recombination. Hendrickson and Duggan, 1976, showed this to be extremely unlikely on statistical grounds and also that altering the multiplicity of infection did not affect the frequency of oversized transductants per p.f.u.

5. Transduction by phage aggregates. If P1 particles aggregate during their manufacture and can then be packed with successive lengths of host DNA, transduction by such aggregates could lead to the recipient cell inheriting several different fragments from the same plasmid. These could then recombine and form a large plasmid. P1 has been shown to aggregate into cruciform structures containing four phages

bound together at the tails (Karamata, 1970). Such aggregates were largely artifacts of purification, however, and were totally non-infective. This does not rule out other as yet undetected forms of aggregation being the mechanism whereby oversized tranductants arise.

B. Transduction of plasmids smaller than P1 headfuls

Production of plasmids by transduction which are significantly smaller than the P1 genome (eg. Tc2, Tc13, Tc18, Table 6.4) may also arise by a number of means, some of which are considered below:

1. Small P1 particles. These have been reported by Ikeda and Tomizawa (1965c) and Walker and Anderson, 1970, but they were too small to transduce DNA over 25 Md. This does not explain the origin of transduced plasmids of around 50 Md.

2. Recircularisation in the new host. This may introduce deletions in the transduced DNA. It is hard to imagine a mechanism for this, however. Recombination does not seem to be involved (see Table 6.2) and illegitimate (IS-mediated) recombination is unlikely as it should occur in the parent strain, producing deletions. These were not detected (see Results).

3. Packaging aberrations. There is much evidence that P1 DNA is packed by a headful mechanism (Ikeda and Tomizawa, 1965a; Yun and Vapnek, 1977; Bächi and Arber, 1977). Generalised transducing particles, however, may be produced by a partial breakdown in the control of the lytic cycle (Harriman, 1972) and packaging of host DNA may therefore be imprecisely regulated.

C. Mechanism of P1 transduction

Two main conclusions can be drawn from the molecular analyses of RA1-1 transductants:

i) There is evidence for a site specific nuclease involved in the packaging of RA1-1 DNA. This was shown by the presence of a novel

4.3 Md. *Bgl*II restriction fragment in digests of five plasmids from phenotypic class 2 (Table 6.1(b), Plate 6.2 and Figure 6.3). This fragment is the result of a specific cut being made in RA1-1. [Such a fragment could be formed by recombination between hot spots on the plasmid molecule when it recircularises in the new host. This seems unlikely, however, for the reasons given in section B2 above.]

The enzyme responsible could be the one postulated by Wall and Harriman (1974) which has the function of recognising specific sites on the P1 genome and may also be responsible for breakdown of host DNA (also at specific sites) during the lytic cycle. The observation (Harriman, 1972) that there are regions of efficient packaging of host DNA supports this. Bächi and Arber (1977) also inferred the existence of a site-specific nuclease responsible for initiating packaging of P1 DNA. [This enzyme is not the P1-coded restriction enzyme (Haberman, 1974) as this has the same six base recognition sequence as *Bgl*II (Roberts, 1977) and so could not be reponsible for initiating P1 packaging at a point between two adjacent *Bgl*II sites on the P1 genome (Bächi and Arber, 1977) nor could it generate the novel restriction fragments seen in *Bgl*II digests of RA1-1 transductants.]

ii) In several cases the P1 transductants of RA1-1 have suffered multiple deletions, or rearrangements, or a combination of both. This is shown by the existence of more than one novel restriction fragment produced by some plasmid transductants (eg. Cm1, Cm3, Cm17, Tc4, Tc6, Tc9, Tc10, Tc18, Plates 6.1-6.3 and Figures 6.2-6.4). A single deletion in a circular molecule can generate only one such restriction fragment, and so these results prove that more complex changes have occurred in these plasmids as a result of transduction.

Any model for P1 transduction must account for the apparent

differences between the transduction of plasmids and that of genes from the chromosome. In the latter case, there is a well-defined upper limit to the length of DNA which can be transduced. This is much less than the size of plasmid which can be packaged. Both the structure and cellular location of the plasmid may be important.

If the topology of RA1-1 is such that it can be packaged while still a supercoiled molecule, then the density of supercoiled DNA is such that more can be incorporated into the phage than with linear DNA. In some cases this allows the whole molecule into the head. Otherwise, any unpackaged, supercoiled DNA is trimmed off. This is accomplished by the "headful" enzyme which can, by slicing off the plasmid DNA not inside the head, introduce several deletions. In order that this enzyme is not active against P1 DNA before it is packaged, it is probably bound to, or a structural part of, the phage particle.

Also involved in packaging is a site-specific endonuclease like that described by Wall and Harriman (1974). The normal function of this protein is to produce that end of P1 DNA which is normally packaged first (Bächi and Arber, 1977).

This model can explain several features of P1 transduction. The difference between transduction of plasmid-borne and chromosomal genes is that the chromosome, either through being wrongly placed or topologically unsuited, is not packaged while still supercoiled. Its incorporation into phage particles is initiated from the specific sites recognised by the site-specific nuclease described above. The amount being incorporated is regulated by the normal phage headful mechanism and is roughly 60 Md. Plasmids, except perhaps when very large indeed, can be at least partly packaged as supercoils. Otherwise, they may first be cut by the site specific nuclease and then packed as a linear molecule of about 60 Md. If there is more than one site on

the plasmid for this enzyme, then a fragment smaller than 60 Md may be incorporated. This is the reason for the different sizes of plasmid transductant. (Packing of supercoiled DNA into small P1 particles may also be involved in generating plasmids of around 50 Md.)

The specific novel fragment seen in digests of plasmids of phenotypic group 2 (see i) above) is produced by the site-specific nuclease. Many of the other novel fragments are produced by the action of the non-specific "headful" enzyme.

Finally, the role of the transductional recipient in the process must be determined. This can be approached by studying the transduction of oversized plasmids into recipients carrying a variety of mutations, particularly in the *rec* genes. Molecular analysis will reveal the importance of host functions in determining the nature of any transductants which arise. [Hendrickson and Duggan (1976) reported that oversized transductants of F'14 were detected only in a *recA*$^+$ recipient.] A parallel study could be made of plasmids the same size as P1 phage DNA, and of smaller plasmids.

Appendix to Chapter 6: The incidence of large P1 particles

Assuming host DNA is packaged randomly into transducing particles, the frequency with which any particular gene is packaged is given by the ratio of the molecular weight of the transduced DNA to the molecular weight of the bacterial chromosome viz:

$$\frac{60 \text{ Md}}{2500 \text{ Md}} = 2.4 \times 10^{-2}$$

Transducing particles form 0.3% of the phage population (Ikeda and Tomizawa, 1965a). Therefore, the frequency with which a transducing particle containing a particular gene is found is given by: $3 \times 10^{-3} \times 2.4 \times 10^{-2} = 7.2 \times 10^{-5}$ per p.f.u.

Transduction of RA1-1 selecting for chloramphenicol or tetracycline resistance seems to demand the inclusion of a least 13.6 Md (or c. 20 genes) of DNA from RA1-1 in the transducing particle (see Results section 4). The frequency with which transducing particles are produced containing this part of RA1-1 is therefore:

$$\frac{7.2 \times 10^{-5}}{20} = 3.6 \times 10^{-6} \text{ per p.f.u.}$$

Roughly one third of the RA1-1 transductants examined were significantly bigger than 60 Md (see Table 6.4). Therefore, large transductants occur at a frequency of c. 1×10^{-7} per p.f.u. If host DNA is incoporated into oversized phage particles at the same frequency as into normal particles, then the incidence of these particles is given by:

$$\frac{1 \times 10^{-7}}{3.6 \times 10^{-6}} = 2.8 \times 10^{-2} \text{ per p.f.u.}$$

Thus roughly 3% of the phage population is oversized. [This calculation assumes that RA1-1 genes are incorporated into transducing

particles at the same frequency as chromosomal genes. It is known that RA1-1 is present in exponentially growing cell cultures at approximately one copy per bacterial chromosome (N. Grinter, unpublished data) but this does not necessarily mean it is equally likely to be transduced. Experiments involving the transduction of chromosomal genes borne by an F', however, show they are acquired by the recipient at a similar frequency to that observed when the same genes are transduced from an F^- donor (Hendrickson and Duggan, 1976) i.e. there is no evidence for the plasmid-borne genes being preferentially packaged.]

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APPENDIX A: Characterization of SmSu plasmids by Restriction Endonuclease Cleavage and Compatibility Testing. Published by The American Society for Microbiology in Journal of Bacteriology, Volume 128, pp. 394-400 (October 1976).

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Characterization of SmSu Plasmids by Restriction Endonuclease Cleavage and Compatibility Testing

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Twelve plasmids carrying genes for streptomycin and sulfonamide resistance were studied for the number and distribution of sites on the plasmid molecules susceptible to cleavage by the restriction endonuclease *Eco*RI. Ten of the twelve were found to have a single cut site, one plasmid (R678) had three such sites, and plasmid PB165, which was isolated as three supercoiled deoxyribonucleic acid species with molecular weights 7.4×10^6, 14.7×10^6, and 21.4×10^6 was reduced to a single (linear) species of molecular weight 7.6×10^6 after cutting with *Eco*RI. We conclude that PB165 forms oligomers in *Escherichia coli* and that the number of copies of these per chromosome is more consistent with a negative than a positive control mechanism for plasmid replication. Compatibility testing of these plasmids showed they all belong to the same incompatibility group, which we designate IncQ, suggesting that they may have come from a common ancestor.

In a previous paper (4) we concluded that the majority of a selection of plasmids conferring linked streptomycin (Sm) and sulfonamide (Su) resistance were very closely related, on the basis of molecular weight comparisons and deoxyribonucleic acid (DNA)-DNA hybridizations. Only 2 of the 12 SmSu determinants studied were markedly different from the rest of the group, namely, R678, which has a molecular weight 60% greater than the others, and PB165, which, instead of one plasmid, consisted of at least three supercoiled species with molecular weights approximately in the ratio 1:2:3.

Another technique for characterizing and differentiating between closely related plasmids involves the treatment of plasmid DNA with restriction endonucleases followed by agarose gel electrophoresis of the DNA fragments generated (17). Small differences in polynucleotide sequences, not detectable by our previous methods, can alter the number or distribution of restriction endonuclease-susceptible sites on a plasmid. We therefore analyzed our SmSu plasmids by this technique. We also hoped to find out whether the plasmids of PB165 are related as oligomers.

The classification of plasmids into incompatibility groups correlates with their polynucleotide sequence relationships (9). Studies on SmSu plasmids by Smith et al. (16) have shown that 19 out of 26 tested belonged to the same incompatibility group, but the recombinant plasmid used by them to test incompatibility was not available to us. We used instead Ap201, which is a transposition (11) derivative of the SmSu plasmid RSF1010, isolated by Heffron et al. (12). Ap201 has acquired a piece of DNA (about 3×10^6 daltons [Mdal]) specifying resistance to ampicillin (Ap), the insertion resulting in the loss of Su^r and a reduction of the level of Sm^r. We suppose that our plasmids are related to RSF1010 because heteroduplex analysis by the same workers has shown that this plasmid hybridizes with R684 (Table 1), leaving only a short sequence of R684 unpaired (12).

MATERIALS AND METHODS

Bacterial strains. Strains of *Escherichia coli* K-12 used were J53 *pro met* (λ) (7), W3110T⁻ *thy dra* (1), and ATCC 14948 *thy* (4). A *thy* mutant of *E. coli* C (*lac met*) was isolated by trimethoprim selection (4).

Bacterial plasmids. Bacterial plasmids used are listed in Table 1.

Isolation of radioactively labeled plasmid DNA. Thymine-requiring strains containing the SmSu plasmids were labeled with radioactive thymine, and their DNA was isolated by CsCl equilibrium centrifugation in the presence of ethidium bromide as previously described (4).

Sucrose gradient sedimentation analysis. Sucrose gradient sedimentation analysis was by centrifugation through freeze-thaw-generated, 5 to 20% sucrose gradients as described previously (4). We calculated molecular weights (M) of DNA molecular species from their relative sedimentation distances (d) from the meniscus, using the relationship (5) $M_1^a = M_2^a \times (d_1/d_2)$, where a, the sedimentation exponent, has been empirically derived by us (manuscript in preparation) as 0.43 for covalently closed circular (CCC) forms, 0.36 for open circular (OC)

TABLE 1. *Bacterial plasmids used*

Plasmid	Molecular mass (Mdal)	Resistance markers[a]	Reference
R300B	5.7	Sm^rSu^r	4
R305C	5.7	Sm^rSu^r	4
R310	5.7	Sm^rSu^r	4
R450B	5.7	Sm^rSu^r	4
R464C	5.7	Sm^rSu^r	4
R676	5.7	Sm^rSu^r	4
R678	9.2	Sm^rSu^r	4
R682	5.7	Sm^rSu^r	4
R684	6.3	Sm^rSu^r	4
R750	5.9	Sm^rSu^r	4
R1162	5.5	Sm^rSu^r	4
PB165	7.4, 14.7, 21.4	Sm^rSu^r	4
R7K-TnC1	33.5	$Ap^rTp^rSm^r$	3
Ap201	9.1	Ap^rSm^r	12
R144*drd*3	65	Tc^rKm^r	14

[a] Resistance marker symbols are: Sm, streptomycin; Su, sulfonamide; Ap, ampicillin; Tp, trimethoprim; Tc, tetracycline; and Km, kanamycin.

forms, and 0.35 for linear (LIN) DNA. For comparison between unlike tertiary forms, we used: $d_{CCC}/d_{OC} = 1.21 \times M^{0.07}$; $d_{OC}/d_{LIN} = 1.11 \times M^{0.01}$.

Transformation. Transformation of *E. coli* C with plasmid DNA was by the method developed by A. E. Jacob and S. J. Hobbs, described previously (4).

Cleavage of plasmid DNA with restriction endonuclease *Eco*RI and agarose gel electrophoresis. The *Eco*RI reaction was carried out at 37°C for 30 min in a reaction mixture containing: about 50 ng of DNA in 10 to 30 μl of TES buffer [50 mM tris(hydroxymethyl)aminomethane, 5 mM ethylenediaminetetraacetic acid, 50 mM NaCl, pH 8]; 10 μl of 0.4 M tris(hydroxymethyl)aminomethane, 0.15 M $MgSO_4$, pH 7.4, and 5 μl of *Eco*RI (a generous gift from S. Cohen); TES buffer to 50 μl. The same mixture without enzyme was used as a control. After incubation, the reaction mixture was placed on ice and 25 μl of 0.05 M ethylenediaminetetraacetic acid-30% sucrose-0.01% bromophenol blue was added. After mixing, 50 μl was layered carefully onto the top of a 1% agarose gel (Seakem Limited) held in glass tubing (5-mm ID) by means of a constricted lower end. Electrophoresis at 3 mA/gel was continued for about 4.5 h in EB buffer [0.5 M tris(hydroxymethyl)aminomethane, 0.2 M sodium acetate, 1.5% (vol/vol) glacial acetic acid, 0.01 M ethylenediaminetetraacetic acid, 0.5 μg of ethidium bromide per ml, pH 7.9], which had also been used to make the gels. Bromophenol blue served as a tracking dye.

After electrophoresis, the gels were removed from their holders, illuminated with ultraviolet light, and photographed through a red filter. Exposure times at f4.5 on Polaroid type 107 film were chosen to give maximum contrast. These were 1 min for gels 15 and 16 and 4 min for the rest.

Incompatibility testing. We used the *E. coli thy* strains in which we have previously isolated each of the 12 SmSu plasmids (4); i.e., the majority of plasmids were in W3110T$^-$, R676 and R682 were in *E. coli* C *thy*, and R1162 was in ATCC 14948 *thy*. We used J53(R144*drd*3)(Ap201) to mobilize Ap201 into the SmSu plasmid-containing strains, selecting for ampicillin resistance and counterselecting the donor J53. Recipient clones were purified on nutrient medium with ampicillin (100 μg/ml) and then tested for the unselected *thy* marker by their failure to grow on DST medium containing 4% lysed horse blood (2). Testing for the presence of a Su^r plasmid in a *thy* strain requires a high (1 mg/ml) concentration of sulfonamide because the thymine (20 μM) necessary for growth acts as a sulfonamide antagonist.

RESULTS

Analysis of DNA fragments produced by *Eco*RI. The banding patterns produced by agarose gel electrophoresis of plasmid DNA before and after cutting by the restriction endonuclease *Eco*RI are shown in Fig. 1. With the exception of R678 and PB165, the SmSu plasmids all gave two bands before enzyme treatment (a faster-running dense band [a] and a fainter band [b] running about half as far as band a). *Eco*RI treatment reduced these two bands to a single one (c).

We correlated the gel bands with the tertiary DNA forms by sucrose gradient analysis of ^{3}H-labeled R300B before and after *Eco*RI treatment, using ^{14}C-labeled R300B DNA as a marker. The results show that the native DNA existed mostly in the CCC form, with a minority in the OC form (Fig. 2a). We therefore conclude that bands a and b in the gels are CCC and OC molecules, respectively. After *Eco*RI cleavage, the DNA sedimented as a single peak (Fig. 2b). Calculation, using the equations given in Materials and Methods, shows that the position of this peak is consistent with its being linear DNA of the same molecular weight as the untreated plasmid (5.7×10^6). Thus, band c is the complete plasmid DNA molecule in linear form, and we conclude that each of the SmSu plasmids apart from R678 and PB165 contains a single site susceptible to *Eco*RI cleavage.

Analysis of R678. R678 has a molecular weight of 9.2×10^6 which is significantly higher than the majority of the SmSu plasmids (5.7×10^6) (4). Gel electrophoresis of untreated R678 DNA gave three bands, presumably representing CCC, OC, and linear DNA forms, the faintest band being linear (Fig. 1, gel 13). After *Eco*RI cleavage, two bands were discernible (gel 14). Sucrose gradient analysis of this cloven R678 DNA, however, gave three peaks (Fig. 3), representing three linear DNA fragments of 7.0, 2.3, and 0.8 Mdal. These three peaks were seen in each of three sucrose gradient analyses of two separate isolations of R678 DNA. Also, the ratios of the radioactive

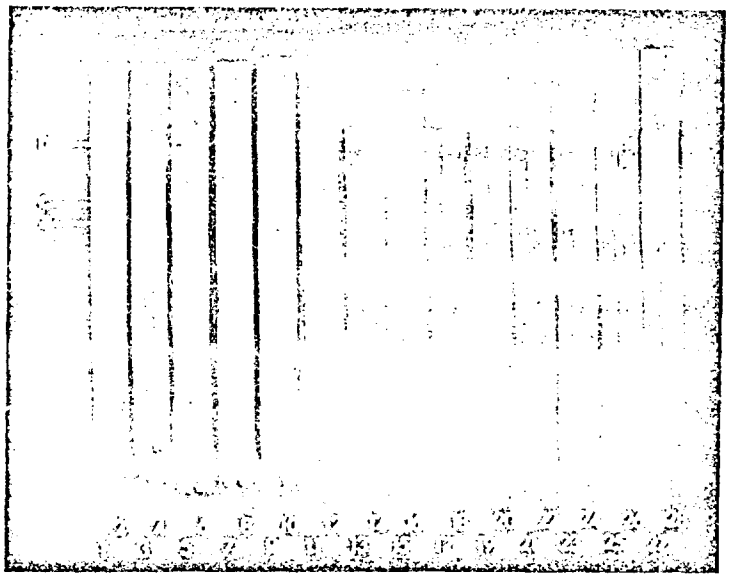


FIG. 1. *Plasmid DNA after electrophoresis on agarose gels in the presence of ethidium bromide. Apart from gels 15 and 16, electrophoresis was continued for 4.5 h. The numbered gels are in pairs containing the same plasmid DNA, the first being native and the second EcoRI-treated DNA as follows: 1 and 2, R300B; 3 and 4, R305C; 5 and 6, R310; 7 and 8, R450B; 9 and 10, R464C; 11 and 12, R676; 13 and 14, R678; 15 and 16, also R678 but subjected to electrophoresis for only 90 min (see text); 17 and 18, R682; 19 and 20, R684; 21 and 22, R750; 23 and 24, R1162; 25 and 26, PB165; 27 and 28, R7K-TnC1. The DNA bands were visualized by illumination with ultraviolet light, which fluoresces with the DNA-ethidium bromide complex. The bands in gels 1 and 2 are the CCC (a), OC (b), and linear (c) tertiary forms of R300B DNA (see text).*

counts in the peaks were proportional to our estimates of their molecular weights. Thus, we are confident that R678 is cut into three DNA fragments by *Eco*RI. We repeated the gel electrophoresis with a fresh preparation of R678 DNA using more DNA than previously (ca. 100 ng) and running it for a shorter time (90 min). Under these conditions, three bands were seen in the gel containing the *Eco*RI-treated DNA (Fig. 1, gel 16). The faint, fast-running band had presumably run off the end in the longer run (4.5 h, gel 14).

Clearly, both sucrose gradient analysis and gel electrophoresis can detect DNA fragments only above a limiting molecular weight. We feel that it is unlikely that we have failed to detect fragments above this limit from the other plasmid DNAs, because the distances travelled by the visible band in each correlate very well with the molecular weights of each entire plasmid (Table 1). There is no evidence of any small DNA fragments in the sucrose gradient analyses of *Eco*RI cloven R300B (Fig. 2b, 3, and 4).

Analysis of PB165. PB165 has been shown to consist of three supercoiled molecules of molecular masses 7.4, 14.7, and 21.4 Mdal (4), and we have suggested that these might be oligomers of a 7.4-Mdal plasmid.

We have also examined a clone of *E. coli* C transformed with DNA from PB165 and found the plasmid DNA to consist of the above three molecular species (data not shown). If this transformation involved the uptake of only a single DNA molecule, then the observed species must be oligomerically related.

Gel electrophoresis of untreated PB165 DNA gave six bands (presumably three CCC and three OC forms; gel 25, Fig. 1). After *Eco*RI treatment only a single band is visible (gel 26). This was confirmed by sucrose gradient analysis, which showed a single linear DNA species of molecular weight 7.6×10^6 (Fig. 4). We conclude that PB165 exists in the cell as oligomers, the monomer having a single *Eco*RI cutting site.

Compatibility testing. As we had established by DNA-DNA hybridization (4) that 10 of this group of SmSu plasmids were very closely related, we expected them to belong to the same incompatibility group. But as R678 and PB165 were less closely related to this group, and especially as PB165 exists intracellularly as oligomers, we considered that they might belong to different groups.

We therefore tested the compatibility of each of the SmSu plasmids with the ampicillin resistance transposition derivative (Ap201) of the

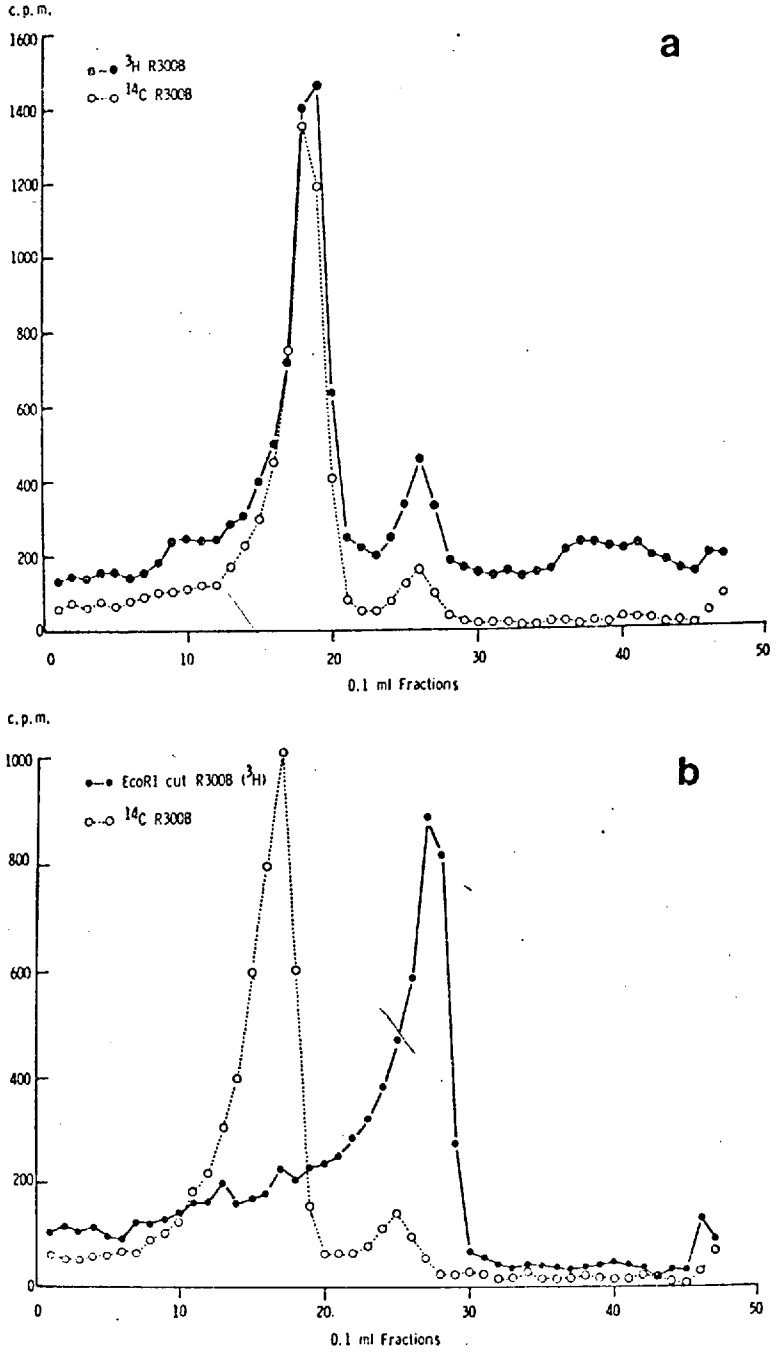


FIG. 2. *Sucrose gradient sedimentation analyses of R300B DNA. (a) Control of untreated ^{3}H-labeled R300B with ^{14}C-labeled R300B. The two peaks for each sample are, left to right, CCC and OC forms, respectively. (b) EcoRI-treated ^{3}H-labeled R300B with untreated ^{14}C-labeled R300B. The single ^{3}H peak represents linear DNA (see text). Sedimentation was from right to left at 100,000 × g for 4 h.*

SmSu plasmid RSF1010. This derivative no longer confers sulfonamide resistance (12). *tra*Ird3 (14) was used to mobilize Ap201 into different strains carrying the SmSu plasmids, selecting for Ap^r recipient clones. After purification, 20 transconjugants from each cross were tested on plates containing ampicillin, kanamycin (Km), or sulfonamide ditches. All 20 were Ap^r and Su^s, from which we conclude that the introduction of Ap201 has eliminated the

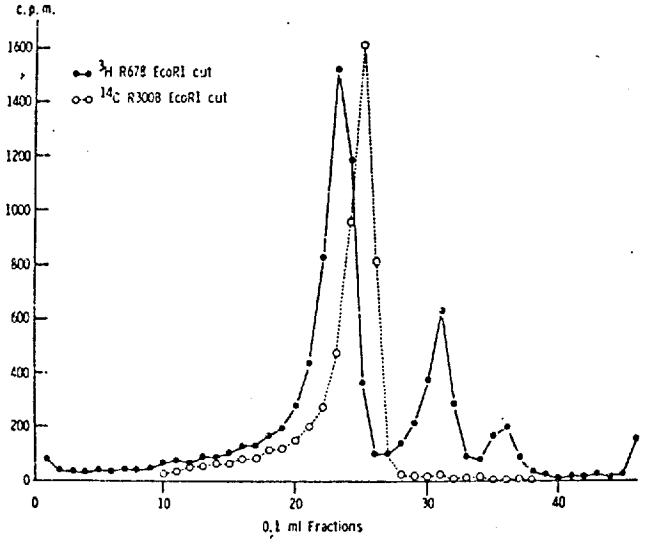


FIG. 3. *Sucrose gradient sedimentation analysis of an EcoRI-treated mixture of ^{3}H-labeled R678 and ^{14}C-labeled R300B DNA samples. R678 is cut into three DNA fragments. Sedimentation was at 150,000 × g for 3 h.*

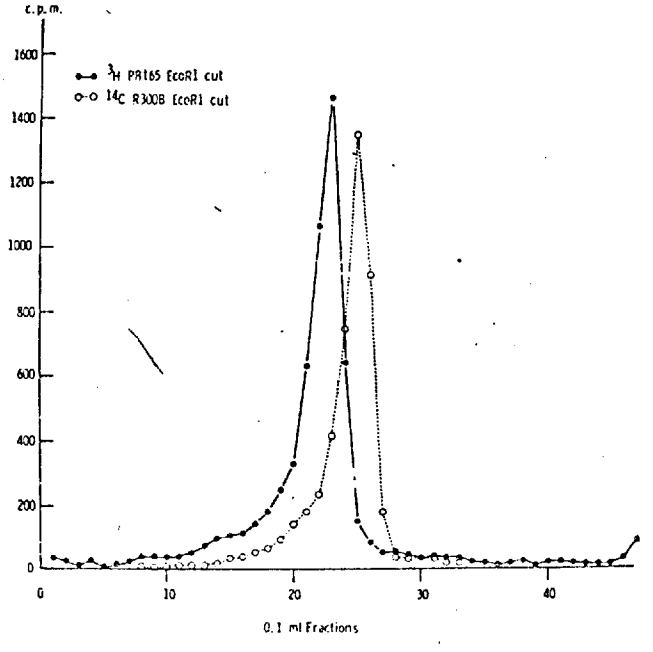


FIG. 4. *Sucrose gradient sedimentation analysis of an EcoRI-treated mixture of ^{3}H-labeled PB165 and ^{14}C-labeled R300B DNA samples. The three DNA species seen in analyses of untreated PB165 (4) have been reduced to a single linear DNA peak of 7.6 Mdal. Sedimentation was at 150,000 × g for 3 h.*

SmSu plasmid. This was true for each of the 12 plasmids tested (Table 1). The majority of the recipients also received R144*drd*3 (as shown by Km^{r}), which belongs to incompatibility group (Inc) Iα (10) and which does not itself eliminate SmSu plasmids (8). We conclude that these SmSu plasmids all belong to the same incompatibility group, which we propose to designate IncQ.

Relationship between electrophoretic mo-

bility and DNA molecular weight. We noted above that the tertiary forms of DNA from the 5.7-Mdal plasmids were separated by gel electrophoresis in the following order from the origin: OC, linear, and CCC. In contrast, Thompson et al. (17) found that under their experimental conditions, the order of DNA tertiary forms was: CCC and then OC and linear together. They studied plasmid DNA of molecular weights 25×10^6 to 65×10^6. To see whether this discrepancy is due to the different molecular weights of the DNAs used, we ran agarose gels with a larger plasmid, R7K-TnC1, which has a molecular weight of 33.5×10^6 and a single *Eco*RI cleavage site (3). A preparation of DNA of this plasmid (shown by sucrose gradient analysis to consist of predominantly CCC molecules, data not presented) gave two bands after electrophoresis, in order from the origin: a dense (CCC) band and a fainter (OC) band that had moved about twice as far through the gel (gel 27, Fig. 1). *Eco*RI-cut DNA gave a single dense band coincident with the OC band of the control (gel 28, Fig. 1). These observations agree with those of Thompson et al. (17), and thus we conclude that the relative electrophoretic mobilities of the three tertiary forms of a plasmid DNA molecule are critically dependent on the molecular weight of the DNA being analyzed. For the 5.7- to 6.3-Mdal DNA, the relative mobilities in increasing order are OC, linear, and CCC. By 9.2 Mdal (R678, gel 13) the linear form has become the fastest, and by 33.5 Mdal (R7K-TnC1, gels 27 and 28) the order is CCC and then coincident OC and linear.

DISCUSSION

We have studied the *Eco*RI cleavage susceptibility of a group of SmSu plasmids that we have previously shown to be related (4). Each of the 10 very closely related plasmids has a single *Eco*RI cleavage site, whereas R678, which is 60% larger and only 45% homologous with the reference plasmid (R300B, one of the ten), has three such sites. Further, as only a single DNA band is seen in the analysis of *Eco*RI-cut PB165 DNA, we have concluded that the three supercoiled molecular species of PB165 are oligomerically related (as their molecular weights suggest). We also conclude that, unless the *Eco*RI site is in the middle of the monomer subunit, these monomers must be joined "head to tail" within the oligomers. We have found that all 12 SmSu plasmids are incompatible with Ap201 and are therefore all in the same incompatibility group (IncQ).

We suggest that the sharing of a common incompatibility group reflects a fundamental common property of these plasmids, namely, their replication control mechanism. This supports our previous contention (4) that these widely spread and very common SmSu plasmids may have originated from a common ancestor.

Whereas we expected the 10 very similar plasmids to be in the same group, we supposed that PB165, with its markedly different molecular weight, DNA homology, and, most important, its existence as oligomers, would probably be in a different incompatibility group. Data from our previous paper (4) show that PB165 has 0.8, 1.3, and 4.1 copies of the trimer, dimer, and monomer, respectively, per bacterial chromosome, which add up to 9 copies of the monomer subunit per chromosome. This matches closely the average of 10 copies/chromosome found for the other SmSu plasmids (especially when one also includes the small amount of tetramer we have observed in some sucrose gradient analyses of PB165 DNA). Thus, PB165 has the same incompatibility function and control of copy numbers (considering the monomer subunit) as the other SmSu plasmids. This is consistent with the model of Pritchard et al. (15) that incompatibility between two plasmids is brought about by their producing an identical or similar "initiation repressor" that controls DNA replication negatively. Each type of initiation repressor gives a characteristic number of plasmid copies per cell, so that two plasmids producing the same repressor within the same cell could not replicate to give more than this characteristic number of copies irrespective of whether these copies exist as monomers or are joined up into oligomers. If segregation at cell division is random with respect to plasmid type, the two plasmids will be observed to segregate, i.e., be incompatible. A negative control model of DNA replication has been supported by recent data (6, 18). The positive control (attachment site) model of Jacob et al. (13) predicts, on the other hand, that the total number of PB165 replicons (as opposed to monomer subunits) would be controlled at the number characteristic of the particular attachment site used by the plasmid for replication. Since PB165 is incompatible with, and thus uses the same attachment site as, the other SmSu plasmids, this model would predict the presence of 10 PB165 replicons/chromosome. Our data of 0.8 + 1.3 + 4.1 giving 6.2 total replicons are less consistent with this model than with a negative control model.

We must also conclude from the above that the formation of oligomers by a plasmid depends upon controls (and replication or recom-

bination mechanisms?) that are different from those regulating its replication; i.e., the initiation control of a plasmid, and its subsequent type of replication, are not necessarily linked. We have observed another example of this phenomenon: RP4 and R751 both belong to incompatibility group P, but whereas R751 produces oligomers, RP4 does not (unpublished data).

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APPENDIX B: Conjugal Transfer System of Plasmid RP4: Analysis by Transposon 7 Insertion. Published by The American Society for Microbiology in Journal of Bacteriology, Volume 133, pp. 43-52 (January 1978).

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Conjugal Transfer System of Plasmid RP4: Analysis by Transposon 7 Insertion

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We have begun an analysis in *Escherichia coli* of the conjugal transfer functions of the broad-host-range plasmid RP4. We have isolated 19 *tra* mutants of RP4, generated by insertion of transposon 7, and mapped their insertion sites by restriction endonuclease analysis. These sites fall into two separate regions on either side of the kanamycin resistance determinant. The transfer rates of the mutants range from 10% of that of RP4 to an undetectable level. Spot tests with the P-1 pilus-specific phages PRR1, Pf3, and PR4 and electron microscopic examination for pili have classified the mutants into several phenotypes consistent with their having normal, retracted, or no pili. Analysis of transient plasmid heterozygotes, created by P1 transduction, divided the *tra* mutants into a minimum of five complementation groups. Some of these groups contain more than one phenotypic class and may represent more than one gene because of the possible polar and deletion effects of Tn7 insertion.

We have recently mapped several genes on plasmid RP4 by using the ability of transposon 7 to insert itself at multiple sites on this plasmid (8). Transposon 7 (Tn7) is an 8.5×10^6-dalton DNA sequence, conferring trimethoprim (Tp) and streptomycin (Sm) resistances and capable of *recA*$^+$-independent transposition from one replicon to another. Its isolation and properties have been previously reported (6). (Tn7 was originally called TnC; see Cohen [14]).

We have mapped Tn7 insertions into RP4 by measuring the sizes of the restriction fragments given by the cleavage of such plasmids with restriction enzymes that have a single available site on RP4 itself, and at least one on Tn7. Insertions giving a particular phenotypic change were found clustered together on the circular map of RP4 at the site, we concluded, of the relevant gene.

Several Tn7 insertion mutants were found to have lost the conjugal transferability of RP4 (8). These mutants, although they gave three different classes of phage sensitivity phenotypes, were found to be clustered into a single small region of RP4. This region, corresponding in size to only one or two genes, was considerably smaller than the *tra* region of plasmid F. We have, therefore, extended this work by mapping more *tra* mutants of RP4. We present evidence here that RP4, unlike F and R plasmids of IncF H, has at least two widely separated *tra* regions.

We have also isolated some mutants with a reduced, rather than undetectable, rate of transfer. These may involve genes that promote conjugation between strains of *Escherichia coli* but are not essential to it, or they may be a consequence of polar effects of Tn7 insertion.

Plasmids, like RP4, belonging to incompatibility group P (P-1 in *Pseudomonas aeruginosa*), confer on their hosts the capacity to produce P-1 pili (9). It is generally assumed that such plasmid-determined pili are involved in the process of conjugal transfer. Various bacteriophages have been shown to adsorb to P-1 pili. PRR1, an RNA phage (26), attaches to the sides of the pili (9). The lipid-containing phages PRD1 (25), PR3, and PR4 (28), which are specific for P-, N-, and W-group plasmids (13), probably attach to the tips (10, 12). The filamentous phage Pf3 (28), specific for IncP plasmids only, is also thought to adsorb to P-1 pilus tips, but this has not been demonstrated. To determine whether or not our *tra* RP4::Tn7 plasmids determined P-1 pili, bacterial strains carrying them were tested for conferred sensitivities to these phages and examined directly in the electron microscope for the presence of pili.

We have assigned the RP4::Tn7 *tra* mutants to a minimum of five complementation groups. P1 transduction was used to form temporary plasmid heterozygotes, which were then scored for their ability to transfer the kanamycin (Km) resistance marker of RP4 to a suitable recipient under conditions of blocked P1 transduction.

We discuss the relationship between complementation groups and *tra* genes.

MATERIALS AND METHODS

Bacterial strains and plasmids. The strains of *E. coli* K-12 and plasmids used in this work are listed in Table 1.

Media. Minimal medium used was M9 (27) with glucose (2 mg/ml), required amino acids (200 μM), and thymine (20 μM). For labeling bacterial DNA, this medium was supplemented with Casamino Acids (Oxoid) at 5 mg/ml, plus [*methyl*-^{3}H]thymine (6.7 μCi/ml) or [2-^{14}C]thymine (1.2 μCi/ml) (Radiochemical Centre, Amersham), and, for *thy*$^+$ strains, deoxyadenosine (1 mM). Nutrient broth was Oxoid no. 2. Media were solidified with 1.2% agar (Oxoid no. 3).

Isolation and testing of Tn*7* derivatives of RP4. Tn*7* insertion mutants of RP4 were isolated as described previously (3); i.e., W3110T-TnC(RP4) was conjugated with J53, and trimethoprim (10 μg/ml)-resistant transconjugants of J53$^+$ were selected. Isolated colonies were purified and tested for the unselected resistance marker of Tn*7*, Sm (5 μg/ml), and those of RP4, ampicillin (Ap) (100 μg/ml), tetracycline (Tc) (10 μg/ml), and Km (25 μg/ml), using antibiotic ditch plates. These purified colonies were screened for conjugal transferability by simple plate matings (7); they were streaked into strain C600 spread over half a plate containing medium selective for Tp^r, Tc^r, or Km^r transconjugants. Clones that appeared transfer deficient were retested by mixing broth cultures of donor and recipient (grown at 37°C without and with shaking, respectively) in a 1:10 ratio, allowing conjugation for 1 h at 37°C without aeration, and then plating onto suitable selective media. Plasmids RP4 and pRP3 (see below) were used as *tra*$^+$ controls in parallel matings.

Sensitivity to phages. Bacteriophage PRR1 was provided by R. Olsen, Pf3 and PR4 were provided by V. Stanisich, and GU5 was provided by J. P. Hernalsteens. High-titer phage stocks were prepared as previously described (13). Spot tests for bacteriophage lytic activity were performed with nutrient plates overlaid with the test strain suspended in soft agar (0.6%) and incubated at 37°C overnight. The observation of any plaques at all was scored as sensitivity.

Complementation analysis of *tra* mutants. The RP4::Tn*7* strains with no detectable transferability were used to make P1 lysates, as previously described (6). These lysates were checked for sterility and titrated for plaque-forming units. Cultures of *tra* strains were grown with aeration in broth with 10 mM $CaCl_2$ to about 5 × 10^8 cells per ml. They were centrifuged, and the cells were concentrated 10-fold in the same medium. Samples (0.1 ml) were mixed with 0.1 ml of the chosen P1 lysate (diluted in P1 buffer [30]) to give a multiplicity of infection of about 1. After 15 min at 37°C to allow adsorption, 0.1 ml of sodium citrate (30 mg/ml in broth) was added. To each suspension was added 0.1 ml of strain C600 (the conjugative recipient), which had been grown in broth with aeration to about 5 × 10^8 cells per ml, centrifuged, and resuspended in one-tenth volume of broth with 10 mg of citrate per ml. After about 2 h at 37°C, 0.3 ml of the mixture was plated on minimal medium containing Km (25 μg/ml), citrate (10 mg/ml), and the nutritional requirements of C600.

Colonies appearing after 2 days of incubation at 37°C were counted. Some of them were purified on MacConkey medium containing Km. Lac$^-$ clones were tested for Tp and Tc resistance with ditch plates and for their ability to transfer Km^r to J53 by using the plate mating technique.

Test for surface exclusion. Exponentially growing cultures of W3110T$^-$(R702) and strains of the RP4::Tn*7* plasmids in J53 were mated in broth for 1 h at 37°C. Dilutions were plated on minimal medium selective for J53 plus 1 mg of sulfonamide per ml. R702 transfer into J53 and J53(RP4-δ1) was scored as surface exclusion (Sex)-negative controls, and transfer into J53(RP4) and J53(pRP3) was scored as Sex$^+$ controls.

Isolation of plasmid DNA. Cultures (7.5 ml) of *E. coli* strains, grown through three to four generations in the presence of radioisotope-labeled thymine, were lysed with Sarkosyl and centrifuged to equilibrium with ethidium bromide and CsCl as previously described (7). Isolated supercoiled DNA was dialyzed overnight against a continuous flow of 5 liters of TNE buffer [50 mM tris(hydroxymethyl)aminomethane–50 mM NaCl–1 mM ethylenediaminetetraacetic acid (pH 7.5)]. Plasmid copy numbers were estimated from the ratio of supercoiled-to-linear chromosomal DNA radioactivity, using the measured molecular weight of the plasmid and taking the *E. coli* chromosome as 2,500 megadaltons (Mdal).

Restriction endonuclease cleavage of DNA. Plasmid DNA in TNE buffer was supplemented with 0.125 volume of a 0.1 M $MgCl_2$–1 M tris(hydroxymethyl)aminomethane buffer (pH 7.5) plus 0.01 volume of the desired restriction enzyme (Miles Laboratories Ltd.), and incubated at 37°C for 1 h.

Sucrose gradient sedimentation analysis. The molecular weights of plasmids and their restriction fragments were estimated by sedimentation, together with suitable reference DNAs, through freeze-thaw-generated, isokinetic (ca. 5 to 20%) sucrose gradients. The preparation of such gradients and calculation of molecular weights have been described previously (7, 8).

Transformation. *tra*$^-$ transposition derivatives of RP4, chosen for electron microscopy, were transferred

TABLE 1. *Bacterial strains and plasmids used*

Strain	Characters[a]	Reference
W3110T$^-$	*thy deoC*	4
W3110T$^-$-TnC	*thy deoC* chromosomally inserted Tn*7*	8
J53	*proA metB*$^-$ (λ)	4
C600	*thr leu thi lacY*	4
CR34-3 *rif*^{r}	*thr leu thi lacY thy deoC rpoB pil*	11
Plasmid		
RP4	IncP Ap^r Tc^r Km^r	15
RP4-δ1	IncP Ap^r Tc^r Tra Sex	17
R702	IncP Tc^r Km^r Sm^r Su^r Hg^r	18

[a] Symbols for bacterial and plasmid characters are according to Bachmann et al. (5) and Novick et al. (22), respectively.

to a strain lacking common pili (CR34-3 *rif*^{r}) by transformation, using plasmid DNA prepared as above and cells made competent according to the method developed by Jacob and Hobbs as described in reference 7. The recipient was, however, grown to late-log phase in broth rather than in P medium. Transformants were selected on nutrient media containing Ap (100 μg/ml), Tc (10 μg/ml), Km (10 μg/ml), or Tp (100 μg/ml), purified on plates containing Km, and then tested on ditch plates for all the RP4::Tn*7* markers. The *thy* and rifampin (*rif*) resistance markers of CR34-3 *rif*^{r} were also tested. (Reduced-transfer mutants were transferred to CR34-3 *rif*^{r} by plate matings and then tested as above.)

All of the RP4::Tn*7* *tra* mutants thus transferred to CR34-3 *rif*^{r} were crossed with C600 to measure their transfer frequency and thus check that this character had not changed. The phage spot tests were also repeated with these strains.

Electron microscopy. Bacterial strains were inoculated from static overnight cultures into fresh broth and grown for 3 h with shaking and then for a further 2 h without shaking. Equal volumes of this culture and PRR1 (ca. 10^{10} plaque-forming units/ml) were mixed in petri dishes to give PRR1-adsorbed pili, and equal volumes of culture and broth were mixed to give untreated control samples. A droplet of each of the mixtures was placed on carbon-coated grids held in forceps (clamped). The forceps were placed in covered petri dishes containing wet filter paper and incubated at 37°C for 10 min. Grids were then washed twice in 0.1 M ammonium acetate and negatively stained in 0.02% sodium phosphotungstate solution. For pilus counts, two grids were prepared from each sample by two different people. Pili on 50 cells were then counted in the electron microscope on each grid by the two people. Cells were counted at random, special care being taken with unlabeled samples not to miss short pili. Numbers presented are the sum of both counts (i.e., 100 cells examined for each sample). Pili were only counted if (i) negative staining was good enough clearly to reveal the cell edge and (ii) the pili projected from the cell edge. Listed counts are therefore comparative only and do not represent the total pili per cell.

RESULTS

Isolation of reduced-transfer mutants of RP4. Tn*7* insertion mutants of RP4 are easily isolated as Tp^r transconjugants from a donor carrying Tn*7* in its chromosome (see Materials and Methods). We have previously shown that about 2% of such transconjugants had lost conjugal transferability (8). (We assume that their initial transfer was mediated by either a cohabiting *tra*$^+$ RP4 or by conjugation mechanisms synthesized before Tn*7* insertion disrupted a *tra* gene.)

We isolated more Tp^r transconjugants from W3110T-TnC(RP4) × J53 conjugations and screened them in a second cross for clones that had a Tp^r transfer frequency that was reduced in comparison with the majority of clones.

Five clones were found with reduced transfer frequencies of all the tested markers. Transconjugants from such matings were found to have acquired all the unselected markers of RP4 (Ap^r Tc^r Km^r) and Tn*7* (Tp^r Sm^r). Thus, the five clones appeared to have plasmids that were reduced-transfer-frequency Tn*7* transposition derivatives of RP4. The plasmids in these strains (see below) were designated pRP76, 89, 90, 91, and 92. (We have previously designated Tn*7* derivatives of RP4 as RP4-TnC followed by a clone number [8]. For brevity and in conformity with the suggestion of Novick et al. [22], we now propose to designate them as pRP followed by the same clone numbers used previously.)

Transfer from reduced-transfer RP4::Tn*7* plasmids is not due to reversion. We considered the proposition that the transfer frequencies observed for the five clones isolated above were a reflection of the reversion frequencies of completely transfer-deficient RP4::Tn*7* mutants. Several transconjugants from each of the five mutants were therefore purified and tested for their conjugation frequency to a secondary recipient strain. We found that these transconjugants retained the characteristic transfer frequency of their parent strains and could not therefore be attributed to reversion.

Transposition of Tn*7* from chromosome to chromosome. In the above isolation, we found two clones that had a markedly reduced Tp^r transfer frequency, but they transferred Ap^r, Tc^r, or Km^r at the same frequency as RP4 itself (i.e., about 10^{-3}/donor per h). Transconjugants selected for one of the RP4 markers carried the other unselected RP4 markers but not Tp^r. We therefore conclude that these two clones carried Tn*7* in their chromosomes together with a free RP4 plasmid. Thus, in a small minority of cases, Tn*7* has been mobilized by a plasmid from its chromosomal site in W3110T-TnC to a chromosomal site in J53. We have noted this phenomenon before (6). We do not know if a plasmid carrying an inserted Tn*7* is a necessary intermediary in this process or not.

Previously isolated but unrecognized reduced-transfer RP4::Tn*7* mutants. Since, in our initial isolation and screening of Tn*7* insertion mutants of RP4, we did not look for reduced-transfer mutants (8), it seemed possible that we had isolated but overlooked such mutants. We therefore measured the conjugal transfer frequency of several mutants, especially those mapping on either side of the *tra* region already established. It was found that pRP26 and 46 (i.e., RP4-TnC26 and 46) had transfer frequencies of about 10^{-5} and 10^{-4} transconjugants/donor per h, i.e., reductions of 100- and 10-fold, respectively, compared to the RP4 or

pRP3 controls. Data on these mutants are therefore included in this paper.

Molecular weights of the *tra* RP4::Tn*7* plasmids. ^{3}H-labeled plasmid DNA was prepared from the newly isolated reduced-transfer RP4::Tn*7* clones and from previously isolated but unmapped *tra* clones (8). Each preparation was cosedimented with ^{14}C-labeled DNA from pRP1 (used as a reference) through a sucrose gradient (data not shown). Each clone was found to contain a single plasmid with a molecular mass of between about 43 and 45 Mdal (Table 2). This mass, as we found previously, is consistent with each plasmid being RP4 (36 Mdal) plus a single copy of Tn*7* (8.5 Mdal) (6). Molecular mass data on the six *tra* plasmids reported previously (8) are also included in Table 2 for completeness.

Restriction enzyme mapping of Tn*7* insertion sites. We mapped the Tn*7* insertion site in each of the *tra* plasmids, using the restriction enzymes *Eco*RI, *Hin*dIII, and *Bam*HI, according to our previous method (8). We established in that paper that each of these three enzymes has one cleavage site on RP4, and that Tn*7* has a single *Eco*RI site and two sites each for *Hin*dIII and *Bam*HI. The relative positions of these cleavage sites were also established (Fig. 1). Thus, one can calculate the site and orientation of each Tn*7* insertion into RP4 from the sizes of the restriction fragments given by cleavage of the RP4::Tn*7* plasmid by using (separately) any two of these enzymes. These data are given in Table 2, the plasmids being listed in the sequence of their calculated Tn*7* insertion sites. This sequence is the best that we could assign from careful assessment and comparison of the restriction data. (As a consequence, Tn*7* insertion sites in pRP28 [Fig. 1], 67, 69, and 77 [Fig. 1 and Table 2], are slightly different [0.1 Mdal] from those previously published [8].) The method does not allow us, however, to establish this sequence unequivocally.

The orientation of Tn*7* in each of these plasmids was found to be the same as in all the (49) RP4::Tn*7* plasmids we have analyzed to date. The orientation is such that the end of Tn*7* closer to its *Eco*RI site (marked with an arrowhead) points clockwise on the map of RP4 as drawn in Fig. 1.

It can be seen from Fig. 1 that the *tra* muta-

TABLE 2. *Molecular masses of the intact and restriction enzyme-cloven RP4::Tn7 tra plasmids*

pRP no.	Molecular mass (Mdal)[a]	*Eco*RI fragments		*Hin*dIII fragments			*Bam*HI fragments			Tn*7* insertion site[b]
		1	2	1	2	3	1	2	3	
46	44.0	36.7	7.8	31.9	10.5	2.1				30.9
92	44.3	36.0	8.3	32.7	9.8	2.0				30.2
76	45.3	35.3	9.2	33.1	9.5	1.9				29.5
90	44.8	35.1	9.4	33.0	9.6	1.9				29.3
89	44.0	34.9	9.6	33.3	9.3	1.9				29.1
67	43.6	34.0	10.5	34.7	7.9	1.9				28.2
69	42.7	34.0	10.5	34.1	8.3	2.1				28.2
68	44.2	33.9	10.6	35.0	7.6	1.9				28.1
74	44.9	33.8	10.7	35.1	7.5	1.9				28.0
66	44.6	33.4	11.1	35.1	7.5	1.9				27.6
81	44.1	33.4	11.1	34.7	7.7	2.0				27.6
75	43.5	33.3	11.2				25.5	18.1	0.8	27.5
77	43.7	33.3	11.2	35.1	7.4	1.9	25.3	18.4	0.8	27.5
79	44.0	33.2	11.3	35.6	6.9	2.0				27.4
78	44.7	33.1	11.4	35.7	7.0	1.9				27.3
26	43.7	33.0	11.5	35.8	6.8	1.9				27.2
91	44.5	23.0	21.5				28.6	15.0	0.9	17.2
80	43.7	22.5	22.0				29.2	14.6	0.7	16.7
82	43.8	22.5	22.0	31.7	10.8	2.0	29.2	14.6	0.7	16.7

[a] The molecular masses of the intact plasmids were measured by cosedimentation through sucrose gradients of each ^{3}H-labeled plasmid DNA with ^{14}C-labeled pRP1 DNA, taken as the reference molecule, with a mass of 45.0 Mdal (8). The restriction fragments of each RP4::Tn*7* plasmid were similarly measured but ^{14}C-labeled RP4 DNA was used as the reference molecule. The RP4::Tn*7* and RP4 DNA samples were mixed before treatment with restriction enzyme. RP4 is cut only once by each of the three enzymes used to give a linear 36.0-Mdal marker each time (8).

[b] The Tn*7* insertion sites have been calculated from the restriction data as previously described (8), i.e., using two sets of restriction data to decide the orientation of Tn*7* and its site in each plasmid. The number in the last column was generally calculated from the *Eco*RI fragment sizes and the position of the *Eco*RI site on Tn*7* as 5.8 Mdal from its right-hand end (Fig. 1 [8]). For pRP91, 80, and 82, the sizes of the *Eco*RI fragments could only be approximately estimated since the two fragments merged into a single peak in the sucrose gradients. We therefore used the *Bam*HI data to calculate their Tn*7* insertion sites (using the *Bam*HI sites on Tn*7* given in Fig. 1). We obtained a standard deviation of 0.2 Mdal for repeated mapping estimates (8).

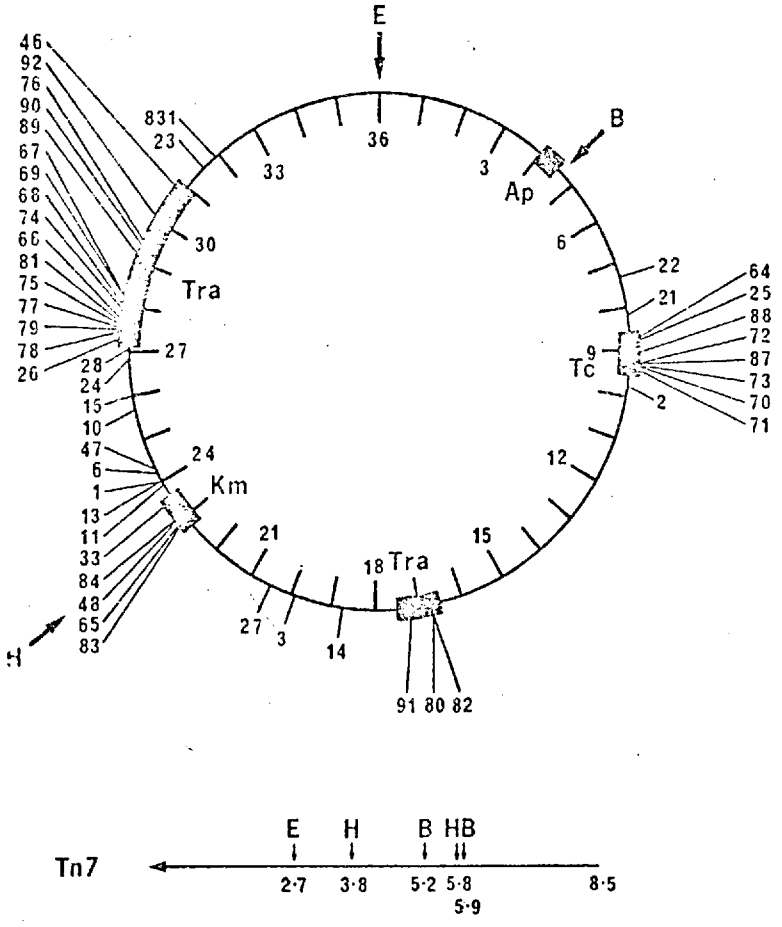


FIG. 1. *Map of Tn7 insertion and restriction enzyme susceptible sites on RP4 together with a diagram of Tn7 showing the approximate locations of its restriction enzyme sites (E = EcoRI, H = HindIII, and B = BamHI). The inner scale of the RP4 circle and the lower scale of the Tn7 map are marked in megadaltons. The Tn7 insertion sites on RP4 are marked with (pRP) clone numbers around the outside of the circle. Insertions that cause phenotypic changes to RP4 are marked on appropriately labeled blocks; those on the Tra blocks are described in this paper. The other insertions and the location of the gene determining Ap^r have been previously published (8). The arrowhead in Tn7 marks the end that we have found always points clockwise in Tn7 insertions of RP4.*

tions map at two quite separate regions, the one we have previously described and a new one at about 17 Mdal.

Plasmid pRP75. We reported previously (8) that pRP75 had a very low plasmid copy number (0.08 per chromosome equivalent compared to an average of 2.0 for the other RP4::Tn7 plasmids), although it was not detectably unstable. The previous mapping data, although unsatisfactory because of the low recovery of plasmid DNA, indicated that its Tn7 was inserted in the 27- to 28-Mdal region. During investigation of the cause of this low copy number, we transduced the plasmid (albeit at low frequency) from this strain (using phage P1 and Km^r selection) to another host and from there back into J53. The plasmid copy number in these strains was within the normal range. The data in this paper were obtained using these transduced strains. The previously approximate Tn7 map site was confirmed, but whereas the original strain conferred no phage sensitivities, the transductants conferred sensitivity to PR4 in spot tests (see below). Data on the cause of the low plasmid copy number in the original strain will be published separately.

Phage sensitivities. We tested all the *tra* mutants for their sensitivity to the phages PRR1, Pf3, PR4, and GU5 with simple spot tests. The results are given in Table 3. As the responses to PR4 and GU5 were always identical, we have not included a separate column for the latter. GU5 is morphologically indistinguishable from PR4 in electron micrographs and is

therefore probably closely related or identical to PR4. They both have a 65-nm-diameter head, which is probably icosahedral, and a 60-nm tail. It can be seen that the plasmids fall into several classes according to the pattern of phage sensitivities conferred.

P-1 pili determined by the RP4::Tn*7* plasmids. We were interested to know how the phage sensitivities related to the number of pili determined by the *tra* RP4::Tn*7* plasmids. Each plasmid was transferred to a strain lacking common pili by transformation or conjugation, as appropriate, and then retested for its resistance markers and characteristic undetectable or reduced-transfer frequency. The pili on 100 cells from each culture were counted in the electron microscope.

From the results given in Table 3 it is clear, firstly, that PRR1 treatment considerably enhanced piliation. Secondly, one can see that there is a general consistency between the results of the phage spot tests and the level of piliation. Thus, of the strains tested that were resistant to all the phages, most had no detectable pili (but 2 of 100 cells of pRP82 had one pilus each after PRR1 labeling). Those with PRR1 resistance but sensitivity to PR4 had detectable pili only after PRR1 adsorbance treatment. Fully phage-sensitive strains had pili under normal growth conditions as well. (It should be remembered that phage resistance indicated by spot test means only that the phage cannot form plaques and does not necessarily imply the complete lack of suitable attachment appendages.)

Complementation analysis. We used P1 transduction to form temporary heterozygotes of the *tra* RP4::Tn*7* plasmids and then, in the presence of citrate (10 μg/ml) to prevent further transduction, tested them for conjugal transferability. Two control experiments were necessary for this technique. Firstly, we checked the effect of citrate on conjugation. In crosses using a *tra*$^+$ RP4::Tn*7* plasmid, we found that citrate did not significantly alter the number of transconjugants (but did reduce the size of the colonies marginally). Secondly, we checked the inhibitory effect of citrate on transduction. We carried out a "complementation" experiment as described in Materials and Methods, but used P1 grown on a plasmid-free strain (P1.R$^-$). A few colonies were found. As these cannot be due to complementation, they must be due to P1 transduction of the *tra* plasmid to the final recipient (C600). Thus citrate does not completely abolish transduction under the conditions we have used. We

TABLE 3. *Conjugal transfer frequencies, phage sensitivities, and P-1 pili determined by the RP4::Tn7 tra plasmids*[a]

pRP no.	Transfer frequency	Phage sensitivity			Untreated pili		PRR1-adsorbed pili	
		PRR1	Pf3	PR4	Pili/cell	Piliated cells (%)	Pili/cell	Piliated cells (%)
46	10^{-4}	S	S	S	—	—	—	—
92	10^{-4}	S	S	S	0.01	1	1.56	65
76	10^{-6}	S	S	S	—	—	—	—
90	10^{-6}	S	S	S	0.05	4	0.74	49
89	10^{-5}	S	S	S	—	—	—	—
67	$<10^{-9}$	S	S	S	0.06	5	0.62	38
69	$<10^{-9}$	R	R	S	0	0	0.01	1
68	$<10^{-9}$	R	R	R	—	—	—	—
74	$<10^{-9}$	R	R	R	0	0	0	0
66	$<10^{-9}$	R	S	S	0	0	0.59	31
81	$<10^{-9}$	R	R	S	—	—	—	—
75	$<10^{-9}$	R	R	S	—	—	—	—
77	$<10^{-9}$	R	R	S	0.04	4	0.15	12
79	$<10^{-9}$	R	R	R	—	—	—	—
78	$<10^{-9}$	R	R	R	—	—	—	—
26	10^{-5}	S	S	S	—	—	—	—
91	10^{-5}	R	R	R	0	0	0	0
80	$<10^{-9}$	R	R	R	—	—	—	—
82	$<10^{-9}$	R	R	R	0	0	0.02	2
Control 3	10^{-3}	S	S	S	0.14	14	0.83	53

[a] Transfer frequency is expressed as transconjugants per donor in nonaerated broth matings at 37°C for 1 h. Phage sensitivities were determined by spot tests. Pili, before and after treatment with PRR1, were estimated by electron microscopic examination of 100 cells. The plasmids are listed in their mapping order (Table 2). —, Not examined.

therefore performed a control experiment using the P1.R⁻ for each of the *tra* plasmids tested and subtracted the number of colonies found from each of the appropriate experiments. The results are shown in Table 4.

Crosses involving the reduced-transfer plasmid-containing strains could only be carried out by using them as P1 donors. They could not be used as P1 recipients (conjugal donors), because the frequency of transduction with a P1 plasmid stock was too low (about 10^{-5}) to expect a measurable increase in their inherent transferability by complementation. This expectation was confirmed. Reciprocal crosses involving these plasmids were, therefore, not possible, nor could we test for complementation between the reduced-transfer plasmids.

We tested about 100 transconjugants from these complementation tests (from crosses involving pRP78, 79, 80, and RP4-δ1) for their subsequent transferability. Only one was found to be Tra⁺ (and Tp^s). Thus, complementation, and only rarely recombination, are responsible for their appearance.

The results in Table 4 show that the plasmids we have isolated belong to a minimum of five complementation groups. Group 1 contains pRP82, 80, and 91; group 2, pRP26; group 3, pRP78 and 79; group 4, pRP77, 75, 81, 66, 74, 68, 69, and 67; and group 5, pRP39, 90, 76, 92, and 46. Because the reduced-transfer plasmids in group 5 could not be tested against one another and they cover about 2 Mdal (3 kilobases), we think that they probably constitute more than one complementation group. We consider below what these groups may mean in terms of *tra* genes.

Surface exclusion. We tested all the plasmids listed in Table 3 for their surface exclusion (Sex) effect on the entry of a second IncP plas-

TABLE 4. *Plasmid complementation data*[a]

pRP plasmid in P1 donor	pRP plasmid in P1 recipient (conjugal donor)												
	67	69	68	74	66	81	75	77	79	78	80	82	RP4δ1
46	*c.* 200									*c.* 900			
92	*c.* 200									*c.* 500			
76	*c.* 40									*c.* 80			
90	*c.* 1,000									*c.* 1,900			
89	*c.* 600									*c.* 1,200			
67	0	0	0	0	0	0	0	0	88				85
69	0	0		0				0					
68	0		0	0				0					57
74	0	0	0	0				0		234			
66	0				0			0	779	702		681	468
81	0					0		0					
75	0						0	0					
77	0	0	0	0	0	0	0	0		98			
79	51								0	0			
78				105				152	0	0		44	22
26	*c.* 900							*c.* 900		*c.* 900		*c.* 900	50
91										*c.* 500		0	
80											0	1	
82										175	0	1	1
RP4δ1	5									10		0	

[a] Crosses were performed as described in Materials and Methods. The plasmids are listed in mapping order (Table 2). The data shown are the number of Km^r colonies given per 10^9 P1 plaque-forming units after subtraction of the appropriate controls. When the P1 donor strain contained a reduced-transfer plasmid, these were: (i) transduction into J53(R⁻) to estimate the conjugal transferability of the resultant transductants (without complementation). This was always less than 1% of the yield from complementing crosses. (ii) Transduction with a P1.R⁻ stock to estimate the number of transductions of C600 due to the transductional recipient acting as a secondary source of transducing particles. This varied between 0 and 10% of the yield from complementing crosses (except for pRP80; see below). In crosses involving plasmids with no detectable transfer only control (ii) was found to be necessary, control (i), when tested, giving no detectable colonies. This is expected from the consideration that the number of secondary sources of transducing particles will be reduced by a factor (ca. 10^5) equal to the primary transduction frequency. When RP4-δ1 was the transductional recipient, its sensitivity to Km made control (ii) impossible (because Km was the selective drug throughout). When the transductional recipient contained pRP80, control (ii) gave consistently higher values than those of the other plasmids. We do not understand the reason for this effect, but it is not due to pRP80 having a higher transduction frequency than usual (about 5×10^{-6}). In contrast, RP4-δ1 has an unusually low transduction frequency (2×10^{-7}), which is responsible for the low numbers in the bottom line of this table. Numbers prefixed by *c.* were calculated from large colony numbers that could only be approximated.

mid, R702. The Sex$^+$ controls, RP4 and pRP3, reduced entry by a factor of about 20 to 50 compared to crosses with J53 or J53(RP4-δ1) (Sex$^-$ controls). None of RP4::Tn*7* plasmids appeared to be Sex$^-$.

The deletion mutant RP4-δ1 has, however, lost its surface exclusion determinant (17). The data in Table 4 show that RP4-δ1 does not complement plasmids in group 1 (pRP91, 80, and 82) but does complement members of the other groups tested. Thus the 9.5-Mdal deletion (8) of RP4-δ1 (which is Km^s *tra* and *sex*) must cover approximately the 15- to 24-Mdal region of RP4. This revision of our previous conclusion about the site of the deletion (δ), together with the consequent probable site of the *sex* gene, is considered below.

DISCUSSION

We have analyzed 19 *tra* mutants of RP4, including six previously reported (8), isolated as Tn*7* insertion derivatives. The insertion sites in these mutants, mapped by restriction enzyme analysis, are located in two quite separate regions of RP4 (Fig. 1). RP4 therefore appears to have at least two *tra* regions rather than one as in plasmid F (2, 3, 23).

The function of the DNA between these two *tra* regions is of interest. We have generated plasmids in vitro, with specific deletions of this DNA (Barth, unpublished data). Together with the evidence on the site of the deletion in RP4-δ1, this shows that there are no genes essential to the viability of RP4 between the two *tra* regions. We therefore speculate that this region carrying the gene conferring Km^r may have been acquired (perhaps by transposition) by a primordial IncP plasmid in response to selective pressure, and that its insertion split a previously contiguous *tra* region.

The plasmid RK2, which is indistinguishable from RP4 in its properties (and may be identical to RP4; J. Beringer, personal communication), has also been the subject of in vitro deletions (16). These experiments, although they define only one *tra* region, are consistent with our map of RP4.

The reduced-transfer properties of some of the RP4::Tn*7* plasmids described in this paper may be due to Tn*7* insertion mutations of genes that promote, but are not essential to, conjugal transfer between *E. coli* K-12 strains. Olsen and Shipley (24) have shown that the transfer frequencies of RP1 in various inter- and intraspecific crosses vary by several orders of magnitude. Some of the *tra* gene products of promiscuous plasmids (like RP1 and RP4) may, therefore, be important in some crosses but not in others. The lack of any insertion mutants in the 28- to 39-Mdal region (Fig. 1) may thus be due not to genetic silence or lack of suitable Tn*7* insertion sites there but to the choice of *E. coli* for the screening of *tra* mutants. The use of other genera may be necessary for a complete analysis of the *tra* regions of RP4.

Alternatively, the reduced-transfer phenotype may be due in some cases to Tn*7* having a polar effect on distal genes within an operon into which it had been inserted. Such polar effects have been reported for Tn*1* (19) and Tn*10* (20). At present we cannot distinguish between the two mechanisms suggested for reduced-transfer mutants; both may be operating.

The unknown polar effects of Tn*7* also limit our interpretation of the complementation groups in terms of *tra* genes. A second complicating factor is the fact that many RP4::Tn*7* plasmids have apparently sustained a deletion of up to 2 Mdal (Table 2). The lack of reversion of such plasmids to Tra$^+$ (Grinter, unpublished observations) is consistent with our previous suggestion that Tn*7* insertion sometimes causes a deletion in the recipient plasmid (8) and that this is at the site of the insertion. This point has been directly confirmed by heteroduplex analysis (G. Engler, M. Van Montagu, and J. Schell, personal communication). Thus, in each of our RP4::Tn*7* plasmids, more than one *tra* gene may be mutant, so that we cannot simply deduce *tra* genes from complementation groups.

The mapping data suggest that complementation groups 2 (pRP26) and 3 (pRP78 and 79) may represent two adjacent *tra* genes (Fig. 1). Group 4 (pRP67, 69, 68, 74, 66, 81, 75, and 77) is complex in that it contains plasmids with various phage sensitivity phenotypes, indicated by spot tests (Table 3), although it covers a length of DNA consistent with being a single gene. Examination of pili determined by members of this group, by electron microscopy, also showed that there were major differences between them (Table 3). Thus pRP69 and 74 have apparently lost the ability to produce pili, but pRP67, 66, and 77 have retained this ability, although they produce significantly fewer pili than the *tra*$^+$ control when not labeled with PRR1. Interpretation of this complementation group must, therefore, await clarification of the complexities due to deletions and Tn*7* polarity effects discussed above.

It should be noted that there were no inconsistencies between the mapping order of the mutants and their clustering into complementation groups.

The marked increase in the number of pili after PRR1 adsorption (Table 3) suggests that P-1 pili are retractile, being "locked" in an ex-

tended position by the adsorbed phages (12). The number of untreated PRR1-adsorbed pili seen by electron microscopy generally reflected the phage spot test sensitivities (Table 3). Visible PRR1 sensitivity apparently required that more than 31% of the cells be piliated after the PRR1 adsorption treatment.

Pf3 and PR4 are both thought to attach to the tips of P-1 pili (10, 12); therefore, we assume that retracted pili may still serve as attachment sites. The results with pRP82, 90, 67, 69, 66, and 77 are consistent with this, although we note that Pf3 and PR4 sensitivities do not always occur together. These phages therefore have different adsorption or infection specificities.

The level of piliation does not reflect the transfer frequency of each *tra* plasmid. For example, strains carrying pRP92 and 90 have about the same number of pili visible but they have a 100-fold difference in transfer frequency. Plasmid pRP67 gives full phage sensitivities and almost normal piliation levels but no detectable transfer (Table 3).

We concluded from the complementation data (Table 4) that the deletion in RP4-δ1 ($Km^s Tra^- Sex^-$) must cover approximately the 15- to 24-Mdal region of RP4. Stanisich and Bennett (29), from a study of deletion mutants, concluded that the Tra and Sex determinants of RP1 are closely linked (about 1 Mdal apart). As RP1 and RP4 are closely related, we predict that the Sex determinant must be at about 16 Mdal. We are now isolating Sex^- RP4::Tn*7* plasmids to test this prediction.

In conclusion, we have shown that at least five *tra* genes, grouped into two separate regions, are involved in the conjugal transfer system of RP4.

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