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ARRANGEMENT AND REARRANGEMENT OF
GENETIC REGIONS ON A BACTERIAL PLASMID

by

MARIA KONARSKA-KOZLOWSKA, M.Sc.

A thesis submitted to the Faculty of
Graduate Studies and Research in partial fulfilment
of the requirements for the degree of
Doctor of Philosophy

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Ottawa, Ontario

June, 1981

"Arrangement and Rearrangement of Genetic Regions on a Bacterial Plasmid" submitted by
Maria Konarska-Kozłowska, M.Sc.
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for the degree of Doctor of Philosophy.

Supervisor

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Abstract

The plasmid content of some strains of E. coli K-12 called RM98⁺ and carrying naturally-occurring R plasmids is shown to include two different conjugative plasmids that are now called pCU1 and pCU2. pCU1 belongs to the incompatibility and conjugative group N. pCU2 could not be identified with any conjugative group so far known among the Enterobacteriaceae. pCU1 and pCU2 showed no detectable DNA sequence homology.

Despite the absence of such homology, the DNA of pCU1 and pCU2 can interact. Derivatives can be obtained which have all the antibiotic markers of the aggregate pCU1 and pCU2 but which neither contain nor segregate pCU2. It is shown that in such strains a DNA fragment of approximately 8.0 ± 0.7 Md has been added to pCU1 concurrently with a Tc^r marker originally present in pCU2 and absent in pCU1. It is suggested that Tc^r in pCU2 is part of a translocatable element. Some RM98⁺ strains of E. coli are such translocation derivatives that contain only an N plasmid. Deletions of pCU1 which result in the loss of Sp^r and Sm^r also arise. Electron microscopic analyses of the denatured DNA of pCU1 or these derivatives revealed several "stem-loop" structures. The loss of Sp^r and Sm^r genes could be linked with the loss of one such stem-loop structure. The distribution of four different stem-loop structures on the pCU1 molecule have been mapped.

pCU1 was studied further as a representative of the N group of plasmids. Electron microscopic measurements of pCU1 DNA and the summation of the molecular weights of fragments obtained after digestion with restriction endonucleases indicate a molecular weight of 26.0 ± 0.3 Md for this plasmid DNA.

Cleavage sites for eight different restriction endonucleases have been mapped using digestion with one or pairs of enzymes. pCU1 has two cutting sites for BamHI, EcoRI, HindIII, and SmaI. KpnI, SalI, PstI and BglII cleave this DNA into more than two fragments. XhoI and XbaI do not cleave pCU1.

In order to localize some of the pCU1-specified phenotypes and to confirm the restriction endonuclease map, a series of deletion mutants has been generated in vitro. Deletions spanning different regions of pCU1 DNA were obtained by digesting the DNA separately with different endonucleases. Genetic and physical analyses of such mutant DNA allowed the construction of a functional map of pCU1. In pCU1, genes for antibiotic-resistance are clustered away from the transfer region. Transfer and sensitivity to bacteriophage IKe are in a common region. A separate 2.7 Md region is needed for plasmid maintenance.

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List of Abbreviations

The genetic abbreviations used are in accordance with the designations suggested by Novick et al. (1976).

Ap	Ampicillin
BSA	Bovine serum albumin
Cm	Chloramphenicol
cpm	Counts per minute
<u>cys</u>	Cysteine biosynthesis
dNTP	Deoxyribonucleotide
E	Electrophoresis buffer
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	Ethylene diamine tetraacetate
EM	Electron microscopy
F	Sex factor
F ⁺	F plasmid bearing
Fig.	Figure
g	Acceleration due to gravity
<u>gal</u>	Galactose utilization
Inc	Plasmid incompatibility group
IS	Insertion sequence
Km	Kanamycin
Md	Megadalton
Mol. wt.	Molecular weight
L broth	Luria broth
u	Micro (10 ⁻⁶)
ug	Microgram

ul	Microlitre
um	Micrometre
P22	Bacteriophage P22
PEG	Polyethylene glycol
pfu	Plaque forming unit
r	Resistance
R	Antibiotic resistance plasmid
R ⁺	R plasmid bearing
R ⁻	Devoid of R plasmid
r-det	Resistance determinant
<u>recA</u>	Genetic recombination
RTF	Resistance transfer factor
s	Sensitivity
SDS	Sodium Dodecyl Sulfate
Sm	Streptomycin
Sp	Spectinomycin
SSC	NaCl; sodium citrate buffer
Su	Sulfonamide
Tc	Tetracycline
TE	TRIS-HCl, EDTA buffer
Tn	Transposon
Tra ⁺	Conjugal transfer proficiency
Tra ⁻	Conjugal transfer deficient
w/v	Weight per unit volume

INTRODUCTION

"Nature is not yet
finished with its
evolutionary experiments."

ARRANGEMENT AND REARRANGEMENT OF
GENETIC REGIONS ON A BACTERIAL PLASMID

The discovery of extrachromosomal genetic elements has opened a new and quickly advancing field in modern biology. J. Lederberg (1952) named such extrachromosomal elements "plasmids". Plasmids can replicate independently of the chromosome and although considered to be dispensable by the cell they are stably maintained and often perform secondary functions that are essential to the cell under certain conditions (Novick et al., 1976; Falkow, 1975). Genes responsible for the production of bacteriostatic agents like bacteriocins and toxins or for enzymes that inactivate or modify antibiotics are often associated with plasmids.

Plasmids may be classified into two major categories: conjugative and nonconjugative (Broda, 1979). A conjugative plasmid can bring about its own transfer by conjugation. In this process which involves cell-to-cell contact the genetic material is transferred from one organism (the donor) to another (the recipient). The classical plasmid F, several colicinogenic plasmids (Col B, V) and a majority of drug-resistance plasmids (R) can bring about the exchange of genetic information in this manner (Broda, 1979). R plasmids are capable of being transferred between various species of bacteria and their spread within bacterial populations has enormous medical and biological implications. Several epidemiological studies have disclosed that bacterial strains may harbour conjugative R plasmids which specify resistance to a number of chemotherapeutic agents currently used for

the treatment of microbial infections (Watanabe, 1963; Falkow, 1975). R⁺ bacteria are then better adapted to changing environments and are more capable of causing bacterial diseases because of their ability to survive during antimicrobial therapy.

The spread of R plasmids is also an important factor in the evolution of new bacterial types (Foster and Kleckner, 1980). Naturally occurring strains of bacteria may carry more than one plasmid. DNA of these plasmids may interact with each other as well as with the bacterial chromosome (Davidson et al., 1975). During such interactions a variety of genetic rearrangements can be created. Deletions, duplications, inversions, transpositions and fusions of genetic regions can all be potentially obtained as a result of plasmid interactions (Bennett and Richmond, 1980).

Two mechanisms for such rearrangements of plasmid DNA have been described (Kopecko, 1980). The first of these involves recombination between regions of extensive homology between two plasmids (or between plasmid and bacterial chromosome). Such events are expected to occur randomly within the regions of homology. In the bacterium Escherichia coli, a functional form of the chromosomal gene recA is also mandatory for such reactions. A second mechanism that has been discovered in recent times can occur in E. coli even in the absence of the recA function and does not require extensive regions of homology (Franklin, 1971). In this process, a discrete continuous region of DNA is thought to

be transposed from one genetic element to another in the same cell (or from one region of a genetic element to another region in the same element). Such discrete regions have been called transposons (or translocons) and some antibiotic-resistance genes have been found to be part of transposons (Kleckner, 1977; Calos and Miller, 1980). It can thus be seen that recombination reactions of these two types together with the interbacterial transfer of conjugative plasmids can lead to rearrangements of genetic regions on the plasmids and thus to the structural evolution of plasmids. This thesis is a study of such rearrangements that occur in a pair of naturally occurring conjugative R plasmids.

Naturally-occurring conjugative R plasmids have been classified into several incompatibility groups on the basis of intracellular incompatibility tests (Datta, 1974). Thus far, most studies on R plasmids have been focussed on those groups of R plasmids that are known to be closely related to the classical plasmid F (Sharp et al., 1973; Kopecko et al., 1976; Rownd et al., 1978). While this is understandable and has allowed a comparison of these R plasmids and their functions to that of F, there has been a need to study R plasmids of other incompatibility groups. For the present study a plasmid of the incompatibility group N was chosen for the following reasons: a) plasmids of this group have been reported to occur frequently in natural isolates of several species of Enterobacteriaceae throughout the world (Hedges et al., 1973; Anderson, 1977; Chabbert et al., 1979),

b) reports have appeared that indicate that some members of this group are genetically unstable, thus suggesting that they may be suitable subjects for the study of gene rearrangements (Ando and Arai, 1979), c) there is evidence to suggest that plasmids of the group N are related to plasmids of two other incompatibility groups P and W; while the nature of this relationship is not known, it has suggested that the N-P-W type plasmids may constitute one interrelated group; if this proves to be correct, it would be important to understand these groups in detail since plasmids of these three groups are extensively distributed in the bacterial kingdom even beyond the Enterobacteriaceae (Datta et al., 1971; Olsen and Shipley, 1973).

The literature review that follows will illustrate how the genetic regions on a bacterial plasmid may be organized and reorganized. The illustrative examples that are used are derived mainly from studies on the classical plasmid F and F-like plasmids. This is because there have been only a few studies on N group plasmids until now and the intensively studied F and F-like plasmids may serve as useful models.

REVIEW OF THE LITERATURE

In many bacterial strains, the genetic information is not exclusively confined to a single DNA molecule, but it is present as a number of distinct molecules of widely differing sizes. All bacterial cells contain the majority of their DNA as a single large self-replicating molecule known as the chromosome (Falkow et al., 1966). In addition to the chromosomal DNA many bacterial species contain extra-chromosomal genetic units known as plasmids (Novick et al., 1976).

Plasmids were first identified in the Enterobacteriaceae, but subsequently have been found in almost every bacterial group where they have been searched for (Cohen, 1976). To date, more than 30 bacterial genera have been found to harbour a variety of plasmids that mediate the expression of diverse phenotypes (Kopecko, 1979).

The essential feature common to all plasmids is a region capable of self-replication as well as the propagation of any genes which are linked to it (Cohen, 1976). Most plasmids are nonessential for cell survival under normal growth conditions and therefore are considered dispensable (Novick et al., 1980). However, very often plasmids play an important role in the survival of their bacterial hosts. Plasmid-borne genes conferring to the bacteria resistance towards many antimicrobial agents such as antibiotics, heavy metals or colicin can be mentioned as examples.

Multiple drug-resistant bacteria were first isolated in Japan in the late fifties. First reports described Shigella strains resistant to chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm) and sulfonamides (Su) (Watanabe, 1963). Soon, bacterial resistance to combinations of new antibiotics were noted, and it was realized that the multiple drug resistant bacteria create a world-wide problem. In other studies, (Watanabe and Fuksawa, 1961b) it was shown that antibiotic-resistance determinants were plasmid linked and furthermore that they could be very efficiently spread among bacterial populations. Watanabe and collaborators provided experimental evidence that the antibiotic-resistance genes were spread during mixed cultivation of resistant and sensitive cells in a process which requires cell-to-cell contact (Wanatabe, 1963). The phenomenon of hereditary exchange in which close contact of cells is necessary has been named conjugation (Lederberg and Tatum, 1946). During conjugation, a sexually differentiated male cell transfers its genetic material to a female bacterium. Sexual differentiation and conjugal ability are dependent upon the intracellular presence of an extrachromosomal genetic element. The first such element endowing the bacterium with fertility was the sex factor F (or F plasmid) (Hayes, 1968; Lederberg et al., 1952). Several other extrachromosomal genetic elements have now been found to also promote conjugation although the F plasmid became a classical prototype with which many other plasmids are being compared (Sharp et al., 1973; Hu et al.,

1975).

Properties of the F plasmid

The fertility plasmid F is a circular DNA duplex, having a molecular weight of 63×10^6 daltons (Freifelder, 1968; Sharp et al., 1973). It can mediate conjugal transfer of DNA. This sex factor has the ability to recombine with the bacterial chromosome at different sites, and can promote the ordered transfer of bacterial genes. In the integrated state the F factor causes the high frequency transfer of chromosomal genes adjacent to it; hence the name Hfr. From the Hfr state, the F factor can excise. Often the excision is not accurate and as a result some of the chromosomal sequences can be incorporated into the F plasmid, which is then referred to as an F-prime plasmid (F') (Davidson et al., 1975). Detailed genetic and physical analysis of F and F' plasmids provided ample information about the molecular organization of this class of extrachromosomal elements. The study of F' factors proved to be particularly important in understanding how the genetic material can be rearranged and how the bacterial plasmids evolved (Davidson et al., 1975; Hu et al., 1975a; Hu et al., 1975b; Sharp et al., 1972; Ohtsubo and Hsu, 1978a, b).

Chromosomal rearrangements caused by F plasmid

An aberrant excision of F plasmid can give rise to two types of F' sex factors. The first type has all the F sequences plus some bacterial genes from both sides of the

chromosomal integration sites. In the second type the F plasmid has lost some of its sequences from either end but has gained some of the adjacent bacterial genes (Davidson et al., 1975; Hu et al., 1975). As a consequence of the latter type of imprecise F plasmid excision, a small segment remains in the host chromosome creating homology for a subsequent F plasmid integration (Curtiss, 1969). The F' factor carries bacterial genes for which the chromosome bears a deletion. If such a F' plasmid is transferred further to a new recipient a partial diploid (diploid with the respect to the bacterial genes carried on the F plasmid) is created. Homology between the bacterial alleles on the F' plasmid and the chromosome results in the integration of this sex factor. Subsequent precise excision of the F element can cause a tandem duplication of bacterial genes.

By means of restriction enzyme and electron microscope heteroduplex techniques (Roberts, 1976; Davis et al., 1971), molecular analyses of F and F' plasmids have resulted in a structural map of the F plasmid (Davidson et al., 1975). An important development from these studies was the identification of specific sequences at various but specific locations on the F molecule. From an analysis of the sites at which chromosomal DNA has been inserted into different F' molecules, Davidson and co-workers were able to determine that these specific sequences act as the recognition sites in F-related recombination phenomenon (Davidson, et al., 1975; Hu et al., 1975). These special sequences which

exist at three locations on the F plasmid molecule have been named as IS2, $\alpha\beta$ and $\gamma\delta$. $\alpha\beta$ was later identified as IS3 (Hu et al., 1975). These sequences belong to a larger class of genetic elements known as Insertion Sequence elements (IS elements). Due to the importance of IS elements in the organization and expression of bacterial genes they deserve a more detailed description.

Some properties of IS-elements

IS elements are well-defined genetic units, usually of constant size that can repeatedly insert into few or many sites in a genome (Campbell, et al., 1979; Novick et al., 1976). The integration of an IS element into a structural gene causes inactivation of the latter. Insertion of IS elements into a gene of an operon not only inactivates that particular gene but also abolishes the expression of the promoter-distal genes. IS-induced mutations are therefore said to be strongly polar (Starlinger and Saedler, 1972; 1976). Polar effect exerted by IS-elements was first observed in the galactose operon of E. coli. Although these gal⁻ mutants could revert spontaneously to wild type, their reversion rate was not enhanced by known mutagens (Starlinger and Saedler, 1972; 1976). Physical studies performed on the DNA of λ transducing phages carrying wild type or mutant galactose operon revealed extra DNA in the affected gal operon (Starlinger and Saedler, 1976; Saedler and Ghosal, 1978).

At the present time, there are several distinct types of IS-elements, recognized. They have been subdivided according to their nucleotide sequences and their length. Table I lists known IS-elements in E. coli (after Saedler and Ghosal, 1978).

IS-elements have not been observed to replicate autonomously (Novick et al., 1976). However, they are able to translocate as discrete DNA segments from one replicating genome to another in the same cell or from one location to another on the same genome. The translocation process occurs by an as yet uncharacterized mechanism that does not require the presence of a functional classical recombination pathway of the host (Starlinger and Saedler, 1976; Kleckner, 1977; Sherratt, 1978; Calos and Miller, 1980).

IS-elements move as single units indicating that the ends of elements are important in recombination events. These recombination events do not require extended DNA homology and are an example of a process called "illegitimate recombination" (Franklin, 1971). "Illegitimate recombination" differs from ordinary recombination in its independence of normal recombination function such as that promoted by recA in E. coli. It also does not require extensive sequence homology between the participating genetic elements. The insertion of IS-elements is also site-specific in terms of the involvement of the ends of the IS-elements. The termini of IS-elements act as "hot spots" in a variety of chromosomal aberrations which occur in the vicinity of the elements.

Table 1
Known IS-elements

<u>Insertion sequence elements</u>	<u>Size (base pairs)</u>
IS1	730
IS2	1350
IS3	1400
IS4	1400
IS5	1400
IS6	115
IS7	60
$\gamma\delta$	5700

Reif and Saedler (1975) described recombinational events involving the DNA sequence IS1 leading to the formation of deletions. The presence of IS1 in the gal operon of E. coli greatly increases deletion-formation in this area of the chromosome. This high frequency of deletion formation is specific for IS1 and is independent of the cellular recA function. Genetic and heteroduplex mapping experiments showed that one endpoint of the deletion is in most cases at the site of the IS1-induced mutation while the other endpoint seems to be variable. IS1 is also responsible for the rearrangement of sequences and deletions that occur in vivo in plasmid pKN101 (Ohtsubo et al., 1978c). The IS2 element also produces deletions in the vicinity of its integration site (Ahmed and Scraba, 1978). Similarly to IS1, IS2-produced deletions have one terminal point located at or near the element. The other endpoint is variable and exhibits no specificity. The complete IS2 element (1350 base pairs) is not needed for the expression of this end specificity. A revertant which retained only 230 base pairs derived from one end of IS2 could produce similar deletions. This supports the notion that the ends of the element are important in the formation of deletions.

Deletion-formation can alter the organization of genomes by bringing together sequences which were far apart before the deletion occurred. The sequences brought together could eventually evolve into operons.

The recognition of IS-elements by virtue of their polar effects implies that they carry signals that influence gene expression (Saedler and Ghosal, 1978). It has been reported that the IS2 element can directly influence the transcription process in E. coli (Chariel et al., 1978). IS2 creates a promoter site when it is inserted in one orientation and a termination site when it inserts in the opposite orientation (Saedler et al., 1974; Saedler and Ghosal, 1978).

IS2 can also cause tandem duplications of the galactose operon in which one set of the gal genes is fused to another promoter allowing constitutive expression of these genes, while the other operon retains the original mutation (Ahmed and Scraba, 1975).

Insertion sequences can also be considered as "mobile regions of homology" (Kleckner, 1977b; Kleckner et al., 1977a). Upon being inserted into different replicons, they can create homologous sequences in unrelated chromosomes, e.g. different plasmids or plasmids and chromosome. These homologous sequences can subsequently be used for gene exchange by classical recombination (Hu et al., 1975; Bennett and Richmond, 1980). Thus, these small units of DNA which may carry enough information to code for two or three proteins can have a profound effect on bacterial evolution. From the evolutionary point of view the ability, for example, of F plasmid to integrate into the bacterial chromosome has important implications. This is because the F⁺ once integrated

can promote the conjugal transfer of the bacterial chromosome to an appropriate recipient and in this way can cause exchange of genetic material between bacteria (Hayes, 1968). The integration of the F plasmid to establish the Hfr state occurs at a restricted number of sites. The DNA sequences for the homology required for the integration of the F plasmid into sites on the chromosome is provided by IS-elements (Hu et al., 1975a). The locations of the insertion sequences on the chromosome are the sites of F integration. It has been estimated by DNA/DNA hybridization that there are about 8 copies of IS1 and 5 copies of IS2 in the chromosome of E. coli K-12 (Starlinger and Saedler, 1976). The idea that IS-elements are active in recombination is supported by the identification of these sequences in F and R plasmids at sites that are important in recombination events (Davidson et al., 1975; Rownd, 1975a, b; Cohen and Kopecko, 1976; Kopecko et al., 1976; Starlinger, 1977; Kopecko, 1980).

A role of IS2 in recombination between plasmid and chromosomal DNA was revealed from the detailed studies of the F' factor F13 (Hu et al., 1975). This plasmid possesses all the sequences of F plus a large segment of E. coli DNA. Hu and collaborators demonstrated that F13 carries an extra copy of the IS2 element which was incorporated together with the bacterial sequences and lies at the insertion site between plasmid and bacterial DNA. The other copy of the IS2 element which is of F plasmid origin lies at the other junction between F factor and bacterial sequences. Thus a copy of

IS2 bridges each junction of plasmid and bacterial DNA. The study of another F' factor F80 gave a similar piece of evidence, demonstrating in this case that the IS3 element also participates in these recombination events (Ohtsubo and Hsu, 1978b). Based on these observations it has been proposed that IS2 and IS3 are vital in the process leading to the formation of Hfr strains (Hu et al., 1975). Similar data have been obtained for the third type of IS sequence observed on the F plasmid, $\gamma\delta$. $\gamma\delta$ element was reported to participate in the fusion of F' factors (Guyer, 1978).

Molecular organization of some R plasmids

Over the past twenty years there has been an increasing amount of study concerning the molecular nature of R plasmids in a variety of bacterial genera (Watanabe, 1963; Clowes, 1972; Helinski, 1973; Sharp et al., 1973; Kopecko et al., 1976).

The initial genetic and molecular studies on R plasmids were carried out using R plasmids which were isolated from Shigella and Salmonella. These plasmids typically conferred multiple drug resistance to chloramphenicol (Cm), tetracycline (Tc), streptomycin (Sm), spectinomycin (Sp), sulfonamide (Su), ampicillin (Ap) and kanamycin (Km) (Watanabe, 1963). Although most of these plasmids were transmissible among members of Enterobacteriaceae, they were not always stably maintained in other species of bacteria, different from the original host.

It was reported that a plasmid, R100 (Tc^R , Sm^R , Su^R , Cm^R), originally found in *Shigella* was easily lost after transfer to *Vibrio cholerae* (Kuwahara, 1972). This plasmid like certain other R plasmids was shown to be unstable in *Salmonella typhimurium* (Watanabe, 1963; Ando, 1979). Derivatives of R100 that lost some or all of their drug-resistances in *Salmonella typhimurium* could be isolated. Analysis of patterns of segregation of drug-resistance genes that occur spontaneously and after conjugal transfer of R plasmids indicated that in most cases the drug-resistance determinants were jointly transferred to recipient cells. Furthermore, the drug resistance genes and the transfer ability were transduced with bacteriophages as a unit when the phage capsid was big enough to accommodate the entire genome of the R plasmid. This indicated that the region encoding resistance to various antibiotics was connected to the region coding for the replication, maintenance and conjugal transfer of plasmids. On the other hand it was also found that *Salmonella typhimurium* strains resistant to Tc, Sm, Su and Ap transferred tetracycline-resistance separately from all other antibiotic resistances. These observations led Watanabe and co-workers (Watanabe, 1963) to propose that R factors are composed of two linked units: a sex factor unit RTF that determines plasmid conjugal transfer proficiency and also Tc^R and a unit carrying other antibiotic resistance genes named r-determinant. This proposal was substantiated by the results of transduction analysis of

some R plasmids utilizing transducing bacteriophage P22. It was noted that the majority of drug-resistant transductants failed to promote conjugation (Falkow, 1975).

An alternative to the model of a transmissible R plasmid consisting of two genetically distinguishable components (RTF and r-det.) that are physically linked was proposed by Anderson (Anderson, 1968).

Anderson studied a strain of Salmonella typhimurium which was resistant to Ap, Tc, Sm and Su. The antibiotic-resistance determinants were transmissible during bacterial matings. Often segregants were obtained that carried transmissible resistance to Sm or Su, Ap, or Tc alone (Anderson, 1968). Using these derivatives as donors in secondary crosses, bacterial cells were isolated that possessed non-transmissible determinants. Electron microscopic analysis and the determination of the molecular weight of the various plasmid derivatives showed that the sizes of Ap, Sm/Su, and transfer factor plasmids were 5.6, 5.6 and 61.0 megadaltons respectively. Preparations of plasmid DNA from cells which carried transferable plasmid and Sm/Su resistance plasmid displayed two types of molecules but these cells did not have any molecules large enough to represent covalent linkage between the transfer component and the Sm/Su components (Milliken and Clowes, 1973). Based on these data, Anderson proposed that in addition to the class of R plasmids that consist of molecules possessing genes for maintenance, conjugal transfer and antibiotic resistance on the same

molecule, there are self-replicating R plasmids that are not self-transmissible, but whose transfer can be promoted by a physically separate conjugative plasmid present in the same cell. Derivatives which were unable to transfer drug resistance(s) could do so if the transfer element was introduced into them.

2 From these early studies of the molecular structure of plasmids it is clear that at least for some R plasmids RTF and r-det. components can exist as a composite structure as suggested by Watanabe or as separate molecules as suggested by Anderson.

• Bacterial hosts influence dissociation and association of composite R plasmids

Certain composite R plasmids that exist as single genetic units in E. coli appear to undergo rearrangement of their components, when transferred into different bacterial species. Many reports describe the dissociation of composite R plasmids into their components upon transfer from E. coli to S. typhimurium (Rownd et al., 1975a, b; Chandler et al., 1978; Humphreys, 1977). These observations demonstrate that even between closely related bacteria such as E. coli and S. typhimurium there are host factors which can influence the structure of plasmids.

The separation and autonomous existence of the RTF and r-det. components of the composite plasmids have become an extensively-studied subject which provided considerable

insight into the molecular structure of dissociable R factors (Rownd et al., 1978). The plasmid NR1 (also known as R100 and R222) and a bacterium Proteus mirabilis became models in these detailed investigations.

The method initially employed involved transferring the plasmid element from E. coli to a strain of Enterobacteriaceae that differed in the average base composition (% G + C) of its chromosomal DNA. This facilitated the separation of the DNA of the plasmid element from the chromosomal DNA, by equilibrium centrifugation in a CsCl density gradient. This technique is based on the fact that the density of DNA is linearly related to the percentage of guanine and cytosine it contains, and this is characteristic for the organism from which it was derived (Schildkraut et al., 1962). Having this in mind, plasmid NR1 was introduced into a Proteus mirabilis strain which has a G + C content which is sufficiently different from the base composition of many R plasmids.

In many cases studied DNA molecules of different plasmids form a single band in a CsCl gradient. This band consists of molecules of the same buoyant density. When NR1 plasmid was isolated from P. mirabilis its DNA consisted of three density species (Rownd 1975a, b). From the analysis of these three species, it was concluded that in P. mirabilis, the largest species represents the complete R factor which can dissociate into two physically and functionally distinctive components corresponding to the transfer factor (RTF) and the region coding for antibiotic resistances (r-det.)

(Helinski, 1973; Rownd, 1975a, b). This finding gave the first molecular evidence for the dissociability of certain R plasmids into component parts. Electron microscopic analyses have shown conclusively that these three molecular species present as a single plasmid in E. coli, dissociate into two units in P. mirabilis (Rownd, 1975a, b; Chandler et al., 1977).

Structural alterations in R plasmid genomes due to the amplification of DNA sequences

It was also observed that if P. mirabilis carrying NR1 is grown in the presence of antibiotics to which its r-det. confers resistance, there is an amplification of r-det. units (Rownd, 1975a, b). This process which is reversible upon transfer of the cells to a drug-free medium has been named "transition". When P. mirabilis carrying NR1 is cultured in drug-free medium for a long period, plasmid DNA forms a single band in CsCl gradients. This DNA species has a molecular weight of 63 Md. After prolonged growth in medium containing antibiotics, a much larger NR1 DNA species is observed. These large molecules consist of many copies of the r-det. joined in tandem with an RTF element (Rownd, 1975a, b). A transition process provides a mechanism of gene amplification. According to the transition model proposed by Rownd (1975a, b; 1977; 1978) both RTF and r-det. units are able to replicate autonomously in P. mirabilis. In drug-free medium the two components are united. In medium containing drugs to which the r-det. component confers

resistance there is a selection for cells having more copies of the r-det. unit as these cells have a higher level of drug resistance. The extra copies of r-det. arise by the disassociation of the composite R plasmid. The amplified r-det. units are joined in a tandem repeat and joined with one copy of RTF component (Rownd et al., 1975a, b). Restriction enzyme analysis and electron microscope denaturation mapping studies support this view (Davidson et al., 1975; Rownd et al., 1978). The addition of multiple copies of r-det. units to an RTF component increases the density as well as the size of plasmid DNA molecules. The reversible amplification phenomenon indicates a plasticity that is inherent in the structure of the NR1 plasmid (Rownd et al., 1975a, b).

Studies involving another composite R plasmid NR84 have shown that the pattern of the increased drug resistance of NR84 in P. mirabilis depends upon the antibiotic which is added as a selective agent (Rownd et al., 1975a, b). P. mirabilis carrying NR84 can also undergo transition. However, in contrast to plasmid NR1 where the entire r-det. region was amplified, in this case upon antibiotic-selection only a part of the r-det. unit showed amplification. Depending on the kind of antibiotic used in the growth medium different segments of the r-det. unit are amplified. Thus, NR84 shows a selective amplification of specific drug resistance genes. This observation suggests that there might be different sites on the plasmid molecule at which the excision and

thus dissociation and gene(s)-amplification can take place (Rownd et al., 1975a, b).

An amplification of drug resistance determinant has also been reported for plasmid pAM α 1 in Streptococcus faecalis (Clewell et al., 1975). Selective amplification of the tetracycline resistance determinant occurs when the S. faecalis/pAM α 1 cells are grown in the presence of this anti-biotic. Yagi and collaborators have shown that the gene amplification involves the generation of tandem repeats of a tetracycline resistance unit (Yagi et al., 1976). In contrast to the NR1 plasmid where the whole r-det. unit undergoes amplification, in the case of pAM α 1 only a 2.65 Mdal. segment of the R plasmid containing the Tc gene is being amplified (Yagi and Clewell, 1976; 1977). Restriction endonuclease analysis of the DNA of this plasmid revealed that 2.65 megadaltons repeats are arranged in tandem on the plasmid molecule (Yagi and Clewell, 1976). Amplification of the DNA segment carrying tetracycline resistance gene(s) can also occur in E. coli (Schöffl and Burkardt, 1979; Schöffl and Pühler, 1979a, b and Mattes et al., 1979) (see p. 36).

Involvement of IS-elements in the dissociation and amplification of gene(s) in composite R factors

It has been proposed that the dissociation and amplification of RTF and r-det. units of composite R factors may be dependent on the presence of specific nucleotide sequences on each structure. These sequences are responsible for a

site-specific recombination event analogous to the integration of the F plasmid into the bacterial chromosome (Rownd et al., 1975a; 1978). Heteroduplex analyses have revealed that IS1 elements are located at each junction of the RTF and r-determinants (Hu et al., 1975; Ptashne and Cohen, 1975; Rownd et al., 1978). Recent heteroduplex analysis and DNA-DNA hybridization experiments performed by Rownd and collaborators have shown that one of the fragments obtained by digestion of NR1 plasmid DNA with EcoRI-restriction endonuclease contains a copy of the IS1 element. The EcoRI fragment containing IS1 lies at the junction of RTF and r-det. unit in NR1 plasmid (Rownd et al., 1978). These experiments strongly suggest that different IS elements or other repeated sequences may interact and mediate R plasmid dissociation and association. The pattern of gene amplification of NR84 plasmid is different from the pattern shown by NR1. NR84 amplifies different fragments of its genome during the transition. In this case, also inverted repeat sequences seem to participate in the drug-resistance amplification process. Most of the NR84 DNA fragments which are amplified appear to terminate at or near regions which contain either IS-elements or inverted repeats (Rownd et al., 1978). The basis of amplification of the Tc-resistance fragment on pAM α 1 plasmid in S. faecalis is also similar (Yagi and Clewell, 1976). Yagi and Clewell (1977) showed that the Tc-resistance determinant on pAM α 1 plasmid is bracketed by homologous sequences of about 400 base pairs that they named as RS-

sequences (recombinational sequences).

Thus, in all of these systems that have been reviewed briefly, amplification is thought to depend upon recombination between homologous IS-elements or IS-like elements. According to the observations made by Rownd et al (1978), Clewell and Yagi (1980), Schöffl and Pühler (1979), and Mattes et al. (1979), dissociation of RTF and r-det. units or the amplification of gene(s) on R plasmids are completely blocked in recA⁻ mutants of E. coli or S. faecalis. This suggests that the repeated sequences of IS-elements provide regions of homology on which classical recombination mechanisms operate.

Organization of some F-like R plasmids

Antibiotic-resistance plasmids R1 and R6 are closely related to plasmid NR1. These two plasmids also exhibit different behaviour in different genera of Enterobacteriaceae. In E. coli all three plasmids exist as composite structures which dissociate into their components in S. typhimurium or P. mirabilis (Cohen and Miller, 1970).

R1, R6 and NR1 are related to the F plasmid. They lead to the production of proteinaceous hair-like structures called pili on the surface of their hosts. These pili agglutinate with F pilus-specific antiserum and absorb F-specific phages (Sharp et al., 1973). F-type plasmids also synthesize a cytoplasmic repressor substance that inhibits DNA transfer and pilus formation by F and other F-like

plasmids (Broda, 1979).

These observations suggest that such phenotypically distinct types of plasmids could have similar or identical genes coding for some or all functions of the transfer region. Therefore, it was important to define a structural basis for these genetic observations.

The electron microscope heteroduplex technique provides an effective method for direct physical mapping of the extent and location of homology between different nucleic acid strands (Westmoreland et al., 1969; Davis et al., 1971). By studying heteroduplex formation between the well-characterized sex factor F and related R plasmids it was possible to identify regions of DNA sequence homology in the respective plasmid genomes (Sharp et al., 1973; Hu et al., 1975b; Kopecko et al., 1976). Five R factors, all closely related to F and therefore called 'F-like' have been studied in detail. They are R1 (Ap^R , Cm^R , Km^R , Sm^R , Su^R), R6 (Cm^R , Km^R , Sm^R , Su^R , Tc^R); R6-5 (Tc^S derivative of R6), R100 (=NR1, R222) (Cm^R , Sm^R , Su^R , Tc^R) and R1d_{rd}19 (Km^S derivative of R1 that is derepressed for conjugal transfer) (Sharp et al., 1973; Hu et al., 1975b; Kopecko et al., 1976).

It was found that approximately 44% of the F factor sequences were homologous with DNA sequences present in R1 and R6 plasmids (Sharp et al., 1972; 1973). The part of the F genome which was homologous with other plasmids included the region in which the genes responsible for transfer were located. The isolated RTF unit of R1 contains all of the

DNA sequences of the parent R1 factor that are homologous to the F plasmid.

DNA sequence homology among the R factors R6, R6-5, R100-1 and R1 was also studied by heteroduplex formation. These F-like R factors all have closely-related sequences (Sharp et al., 1973). The heteroduplex method was also used to map the resistance determinants on some of these R plasmids (Sharp et al., 1973; Kopecko et al., 1976). By analyzing the heteroduplex molecules formed by annealing R1 and its antibiotic sensitive deletion mutant (RTF), it was possible to localize the genetic regions on R1 DNA specifying drug-resistance. Furthermore, by comparing the R6-5 plasmid which specifies resistance to Km with R1drd19 which is Km-sensitive an extra segment of DNA on R6-5 was detected, the rest of the regions of the plasmids being homologous. Km-resistance was mapped within the extra DNA on R6-5. In contrast to the other resistance determinants, Tc was found to be in the RTF region (Sharp et al., 1973; Kopecko et al., 1976).

Presence of IS-elements on F-like R plasmids

Insertion sequences were observed in several places on those R plasmids that have been studied. Heteroduplex experiments between the plasmid R6 and one strand of the DNA of λ phage carrying the IS1 element, showed that IS1 occurs on R6 at the junction of RTF and r-det. (Hu et al., 1975b). From previous heteroduplex analysis (Sharp et al., 1973), it appears that IS1 occurs at the same junction in R6-5, R100-1

and R1 plasmids. Heteroduplex molecules with the DNA from a phage carrying IS2 showed that IS2 also is present in these plasmids (Hu et al., 1975b). Repeated sequences were also found in other regions of these R plasmids, sometimes associated with the change in drug resistance (Sharp et al., 1973).

All of the F-like R factors studied showed a high degree of homology within the transfer region. The differences observed among them were assigned mainly to the region containing antibiotic resistance genes. On the basis of these observations it was proposed that R1, R100 and R6 are descendants of a single plasmid which subsequently acquired or lost some of the drug resistance genes. Electron microscope studies of DNA sequence relationship between bacterial plasmids provided the earliest molecular evidence that certain plasmids may be formed by site specific association of precisely defined segments of DNA (Cohen and Kopecko, 1976; Kopecko and Cohen, 1975).

Transposable elements associated with drug-resistance determinants

Insertion sequences are the simplest examples of the large group of prokaryotic translocatable elements (Kleckner, 1977; Calos and Miller, 1980). Recently it has been shown that many plasmid specific genes reside on translocatable units called "transposons" (Kleckner, 1977 and Calos and Miller, 1980). Transposons were originally distinguished from IS-elements because unlike the latter, they carry detect-

able genes, often genes conferring antibiotic resistance (Hedges and Jacob, 1974; Heffron et al., 1975; Kopecko and Cohen, 1975; Berg et al., 1975; 1977; Bennett and Richmond, 1976; Reubens et al., 1976; Barth et al., 1976). There is a close relationship between IS-elements and transposons. Transposons often terminate in inverted or direct repeats and these repeat-segments are IS or IS-like elements (MacHattie and Jackowski, 1977; Clerget et al., 1980). The experiments of Sharp et al. (1973) provided the initial description of inverted repeats on plasmid DNA. Such inverted repeats, which consist of DNA segments repeated on the same strand of DNA, were observed in several of the plasmids studied. During heteroduplex analysis rapid intrastrand annealing of the inverted repeated sequences resulted in the formation of a single-stranded loop, which contained the duplicated sequences as a double-stranded stalk. Heteroduplex mapping data involving plasmids carrying different drug resistance determinants have shown that in some cases the genes coding for two different kinds of antibiotic resistance (tetracycline and kanamycin) are located within the vicinity of these inverted repeats (Sharp et al., 1973).

The repeated sequences of the transposable elements serve as "hot spots" in the translocation process. It has been documented that "transposons" retain their functional integrity during repeated cycles of translocation. Several different and distinguishable transposons have been followed through multiple cycles of translocation under conditions

when homology-dependent interactions between the element and the target site were excluded. For example, Tn10 (a tetracycline resistant transposon) has been shown to translocate from its original position on an R plasmid to the genome of phage P22, from P22 to a leucine structural genes of the Salmonella typhimurium chromosome and from the S. typhimurium leucine operon into phage λ (Kleckner, 1977). Acquisition of tetracycline resistance by phages P22 and λ and by the R plasmid has been seen as a specific stem-loop structure defined by heteroduplex analysis (Kleckner et al., 1975; Botstein and Kleckner, 1977).

Analogous experiments have shown that other elements such as Tn5 (Km resistant element), Tn9 (chloramphenicol resistant element), Tn1, Tn2, Tn3 (Ampicillin resistant elements) and Tn4 (Ap^R , Sm^R , Su^R -element) are capable of recA-independent movement as discrete units to many sites.

Most of these elements, like the IS-elements, have been shown to cause mutations by insertion into known structural genes. Insertions within operons are polar (Hedges and Jacob, 1974; Heffron et al., 1975; Kopecko and Cohen, 1975; Berg et al., 1975; 1977; Kleckner et al., 1975; Bennett and Richmond, 1976; Reubens et al., 1976; Barth et al., 1976). Transposons are larger than IS-elements. They range in approximate size from 4500 base pairs (Tn1, 2, 3) to 20,000 base pairs (Tn4) (Heffron et al., 1975a, b; Kopecko et al., 1976). Table II contains a list of some transposable elements (after Calos and Miller, 1980).

Table 2

Procaryotic transposons

Transposon	Marker	Length (base pairs)	Inverted Repeat	Comments
Tn1 Tn2 Tn3	ampicillin	4,957	38	full sequence of Tn3 known
Tn4	ampicillin, streptomycin, sulfonamide	20,500	short	contains Tn3
Tn5	kanamycin	5,400	1500	sequence of ends known
Tn6	kanamycin	4,200	not detectable with electron microscopy	
Tn7	trimethoprim, streptomycin	14,000	not detectable with electron microscopy	
Tn9	chloramphenicol	2,638	18/23	full sequence known, flanked by direct repeats of IS1
Tn10	tetracycline	9,300	1400	sequence of ends known
Tn204	chloramphenicol, fusidic acid	2,457	18/23	full sequence known, flanked by direct repeat of IS1
Tn402	trimethoprim	7,500	not detectable by electron microscopy	

Tn501	mercuric ions	7,800	38	sequence at ends known
Tn551	erythromycin	5,200	35	in <u>Staphylococcus aureus</u> (gram positive) sequence of ends known
Tn554	erythromycin, spectinomycin	6,200	not determined	in <u>S. aureus</u>
Tn732	gentamicin, tobramycin	11,000	not determined	
Tn903	kanamycin	3,100	1050	sequence of ends known
Tn917	erythromycin	5,100	short	in <u>Streptococcus faecalis</u> (gram positive)
Tn951	lac	16,600	short	
Tn1681	heat stable enterotoxin	2,088	768 (IS1)	full sequence known
Tn1721	tetracycline	10,900	short	sequence of ends known
Bacterio- phage Mu		38,000	absent	sequence of ends known

The different transposons differ in the degree of specificity which they display. Some appear to insert randomly whereas others have preference for particular sites (Berg et al., 1975; Grindsted et al., 1978; Heffron et al., 1975; Kleckner et al., 1975; 1979a; Tu and Cohen, 1980). They have also been implicated in different recombination events other than translocation such as precise and imprecise excision; deletion, duplication, inversion of adjacent chromosomal sequences and integration of F into the bacterial chromosome (Botstein and Kleckner, 1977; Chumley et al., 1979; Kleckner et al., 1978; 1979; MacHattie and Jackowski, 1977; Ross et al., 1979a, b; Kehoe and Foster, 1979; Nisen et al., 1977; Brevet et al., 1977).

Mutations caused by transposons can revert to a non-mutated phenotype with the concomitant loss of the associated drug resistance marker. However, excision of the transposable element is not always precise (Kleckner et al., 1975; 1978; Ross and Kleckner, 1979a, b). The well studied tetracycline-resistance transposon Tn10 often promotes deletions in regions adjacent to it. In some of these deletions, a small piece of the element is left behind (Ross and Kleckner, 1979a, b). Such deletions are referred to as "imprecise excisions" of Tn10.

Translocatable elements were also reported to promote duplications via illegitimate recombination. Insertions of many elements result in small duplications of non-transposon DNA (9 or 5 base pairs) at their target site (Calos et al., 1978; Grindley, 1978).

Structural and Functional anatomy of a transposable element

The most complete current picture of the structural and functional anatomy of a transposable element concerns Tn3, a transposon which confers resistance to ampicillin. This drug resistance is associated with the enzyme β -lactamase. Hedges and Jacob (1974) demonstrated that plasmids gaining this marker show an increase in molecular weight of 3×10^6 daltons. Heffron et al. (1975) showed that a sequence of this length is common to a variety of plasmids specifying ampicillin resistance. DNA sequencing analysis revealed the transposon as an insertion bounded by short inverted repeat sequences of 40 base pairs (Heffron and McCarthy, 1979). Translocation of ampicillin resistance to other replicons is independent of recA function of E. coli (Bennett and Richmond, 1976; Reubens et al., 1976). The translocation of Tn3 to the chromosome is much less frequent than is translocation to other plasmids (Kretschmer and Cohen, 1977). Plasmids already containing Tn3 are much less susceptible to the translocation of another ampicillin transposon than are related Tn3-free plasmids. However, other cohabiting plasmids are still susceptible (Robinson et al., 1977). Furthermore, acquisition of a transposon by a plasmid was far more frequent than its demonstrable loss from the donor plasmid (Bennett et al., 1977). One possibility is that transposition involves duplication of the transposon (Shapiro, 1979; Fennewald and Shapiro, 1979), another is that excision normally results in the destruction of the donor replicon

(Bennett et al., 1977).

Tn3 has been dissected with a series of mutations generated in vitro, beginning with a set of deletions produced by exonuclease III and endonuclease SI (Heffron et al., 1977), and continuing with a collection of mutations made by insertion of a synthetic EcoRI site (Heffron et al., 1978). Several of the mutations produced affected Tn3 transposition (Heffron et al., 1979). From the analysis of these mutants it was possible to identify three proteins whose synthesis is directed by Tn3. In addition to β -lactamase the transposon codes for a transposase and a repressor which regulates the transposase. Several studies indicate that the repressor is self-regulated (Gill et al., 1979; Chou et al., 1979; Dougan et al., 1979). The three Tn3-encoded proteins have been synthesized in minicells and isolated on gels (Dougan et al., 1979; Gill et al., 1979). The repeated ends and also some internal regions are necessary for transposition. Tn3 contains three sites required in cis for normal transposition (Gill et al., 1978; Heffron et al., 1979). The two ends of the transposon are necessary and a site localized in the middle of the transposon is needed. If this site is deleted the donor and recipient plasmids are fused (Gill et al., 1979; Arthur and Sherratt, 1979).

Other transposons also code for functions required for their own transposition. Studies with derivatives of kanamycin-resistance transposon (Tn5), carrying substitutions made in vitro indicate that Tn5 encodes trans-acting trans-

position functions in inverted repeats (Meyer et al., 1979; Postle and Reznikoff, 1979; Rothstein, 1980). Several polypeptides encoded by inverted repeat portions of Tn5 have been identified. The evidence indicates that one of the inverted repeat units differs slightly from the other and contains a promoter for the kanamycin resistance determinant while the other inverted repeat unit is dominant in supplying transposition functions. The polypeptides from the latter repeat of Tn5 are absolutely required for Tn5 transposition (Rothstein et al., 1980). The dissimilarities between the two inverted repeats of Tn5 are caused by a single base pair change. This change is shown to be sufficient to explain all the functional differences between the two inverted repeats of Tn5 (Rothstein and Reznikoff, 1981).

It has been proposed that the repeated sequences of the ends of transposons originated from IS-elements. In some cases the ends of transposons were identified with known IS elements. Examples would be the chloramphenicol resistance transposon, Tn9 or the kanamycin-resistance transposon, Tn2350, both of which carry IS1 sequences at their ends (MacHattie and Jackowski, 1977 and Clerget et al., 1980). It has further been speculated that during evolution one of the two IS-elements in a transposon could undergo an alteration without losing the ability to transpose. This alteration would be in the form of a large deletion leaving short repeats as is found for Tn3 (Heffron et al.,

(1977) or the change could be more subtle as in Tn5 (Rothstein and Reznikoff, 1981).

Similar observations have been made for the ends of Tn10. In this case, in vitro-generated deletions show again that the inverted-repeats encode a trans-acting function required for transposition and again only one of the inverted repeat unit is required for the transposition (Foster et al., 1981).

There is some evidence that the ends of Tn10 can also behave as independent IS-elements. In a Tc^S-derivative of R6 plasmid (R6-5) a copy of the inverted repeat sequence flanking Tc resistance was inserted into the tetracycline gene(s) to cause its reversible inactivation (Sharp et al., 1973; Foster and Kleckner, 1980).

Further homology between IS-elements and transposons can be extended to the gene amplification phenomenon. As described earlier (see p. 24) it has been shown that in some cases IS-elements are directly involved in recA-dependent gene amplification. Two transposable elements Tn1771 and Tn1721 are able to amplify the tetracycline resistance gene associated with them (Schöffl and Burkardt, 1979c; Schöffl and Pühler, 1979a, b; Mattes et al., 1979; Schmidt et al., 1979). Tandem repeat copies of a transposon segment specifying tetracycline resistance were observed. In the case of Tn1771, the authors suggest that the intramolecular amplification is due to a small directly repeated DNA sequence flanking the amplifiable region of the transposon (Schöffl

and Pühler, 1979a, b).

Tn1771 contains three short repeated sequences. Two of them flank the entire transposon as inverted repeats. These inverted repeats have been proposed to participate in the recA-independent translocation process. The third repeated sequence in the middle of the element is thought to be involved in the recA-dependent amplification phenomenon (Schöffl and Pühler, 1979a, b). These observations suggest that at least some transposable genetic elements can also be subject to reorganization. Furthermore, the inverted repeat sequences connected with transposons and IS-elements can be envisioned to participate in two types of recombination events. Transposition and insertion of these elements has been shown to occur in recombination-deficient (recA) bacterial hosts by a process which does not require extensive homology between two interacting chromosomes. On the other hand, there is also ample evidence which indicates that the repeated sequences of transposable elements serve as homologous sequences upon which the general recombination mechanisms can act (Rownd et al., 1978; Yagi and Clewell, 1980; Schöffl and Pühler, 1979).

Exchange of genes by general recombination is a well established phenomenon in bacteria (Hayes, 1968). R plasmids are certainly a subject of this type of recombination, which involves a random physical breakage and reciprocal exchange of DNA in an area of extensive genetic homology. Many examples of plasmid-plasmid interaction, as well as

recombination events between plasmids and other replicons has an absolute requirement for a functional recA gene in the host (Bennett and Richmond, 1980; Falkow, 1975).

In general, plasmids which have a significant portion of their DNA sequences in common are said to be incompatible (Novick et al., 1976). Such incompatible plasmids cannot stably coexist in the same bacterial cell without selective pressure. In many instances however, R factors of the same incompatibility group may recombine to produce a recombinant plasmid molecule which then can survive the selective conditions that were imposed (Datta, 1977). Thus, recombination between closely related plasmids like F and F-like R factors have been observed (Falkow, 1975; Bedbrook and Ausubel, 1976).

The fact that extensive sequence homology is needed for classical recombination (recA-dependent) to occur, ensures that the recombination between plasmids of the same incompatibility group is relatively common. Conversely, pairs of compatible plasmids tend to have relatively little sequence homology and to recombine poorly (Bennett and Richmond, 1980). Classical recombination between DNA molecules is a reciprocal process (Hayes, 1968). The information initially present in the two molecules is conserved following the completion of the process. When plasmids, e.g. circular molecules, are involved, the reciprocal nature of the recombination may lead to the fusion of the two DNA molecules to form a single circular

structure containing information present in both replicons. Fusion of homologous plasmids has been reported (Bennett and Richmond, 1980; Bedbrook and Ansubel, 1976). This remarkable structural and genetic diversity of plasmids seems to be shaped in part by the general recombination, which produces more delicate changes, and in part by the "illegitimate recombination" which triggers gross alterations in plasmid genomes.

In this review of the published literature I have tried to show that the structure of an R plasmid is not stable, but is subject to all kinds of recombinational events operating inside the bacterial cell. These events which are said to be "general" or "illegitimate", can lead to various kinds of rearrangements of the DNA sequences and genetic regions constituting plasmids. To illustrate these changes, and the general organization of R plasmids, I selected only a few better-studied examples from the literature. As the existing amount of published information concerning translocatable elements is vast, only those examples that are directly relevant to antibiotic-resistance plasmids and those that have been well-studied have been considered in this review.

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages

Bacterial strains and plasmids used or derived are listed with their properties in Tables 3 and 4. The bacteriophages used and their sources were as follows: R17, W. Paranchych; If, E. Meynell; PRD1 and PRR1, R. Olsen; Pfl, V. Stanisch; IKE, this laboratory; IKE hl (a mutant of IKE with a host range extending to P Incompatibility Group plasmids), R.B. Grant; P22 int (an integration-deficient mutant of P22), P. Hartman, λ b221c1857, D. Berg.

Media and chemicals

Antibiotic Medium 3 (Penassay Broth) of Difco Laboratories with or without 1.5% agar was used as the standard organic medium to which antibiotics or inhibitors were added as required. The concentration of the various antibiotics or inhibitors used in the plates was ampicillin (Ap), 30 ug/ml; kanamycin (Km), 30 ug/ml; spectinomycin (Sp), 50 ug/ml; streptomycin (Sm), 50 ug/ml; tetracycline (Tc), 20 ug/ml; and nalidixic acid, 50 ug/ml. The minimal agar medium was that of Davis and Mingioli (1950) to which sterile supplements were added at the following final concentrations after sterilizing them separately by filtration: L-amino acids, 40 ug/ml, thiamine, 1 ug/ml; thymine, 2 ug/ml; glucose, 0.2% (w/v). Restriction endonucleases were purchased from New England Biolabs.

Abbreviations: nal, nalidixic acid; str, streptomycin; km, kanamycin; r or s resistance or sensitivity; arg⁻, his⁻, met⁻, pro⁻, pyr⁻, pur⁻, thy⁻, thi⁻, leu⁻, cys⁻, aro⁻, thr⁻, dependence on arginine, histidine, methionine, proline, pyrimidine, purine, thymine, thiamine, leucine, cysteine, aromatic amino acid, threonine; gal, galactose; mal, maltose; mtl, mannitol; lac, lactose; λ bacteriophage lambda; RecA⁻ general recombination deficiency; rk⁻, restriction deficient; rm⁻, modification deficient; supE, suppressor E.

Table 3
Bacterial strains*

Strain	Relevant marker in the strain	Source	Reference
C600	thr ⁻ leu ⁻ thi ⁻	S.N. Cohen	Bachmann (1972)
WD00	thy ⁻ thi ⁻ mal ⁻ gal ⁻ str ^r	T. Ogawa	Ogawa (1975)
CR34	thr ⁻ leu ⁻ thy ⁻ thi ⁻ str ^r supE	F. Bonhoeffer	Bonhoeffer (1966)
CR34/pCR1	thr ⁻ leu ⁻ thy ⁻ thi ⁻ str ^r supE km ^r	J. Carbon	Covey <u>et al.</u> (1966)
J53	pro ⁻ met ⁻	N. Datta	Bachmann (1972)
490	thr ⁻ thy ⁻ thi ⁻ leu ⁻ recA ⁻ rk ⁻ rm ⁻	D. Thomas	
AT2455	thi ⁻ cys ⁻ mal ⁻ λ ⁻	B. Bachmann	Bachmann (1972)
AT2472	aro ⁻ thi ⁻ λ ⁻	B. Bachmann	Bachmann (1972)
C08	arg ⁻ his ⁻ lac ⁻ met ⁻ mtl ⁻ pro ⁻ pur ⁻ pyr ⁻ str ^r thy ⁻ nal ⁻	K.B. Low	Bachmann (1972)
W1485	nal ^r	K.B. Low	Bachmann (1972)
LT2	cys ⁻	J. Brenchley	

*The strains listed above are all free of the plasmid pCU1 or its derivatives. In addition to the strains listed here, a large number of other derivatives carrying pCU1-related plasmids were constructed and used. Such strains are referred to in the text by adding the name of the plasmid in parenthesis following the name of the plasmid-free strain. For example strain C600 (pCU1) is a derivative of C600 containing pCU1. Unless otherwise indicated in the text all plasmid positive strains were constructed by conjugation.

Table 4

Plasmids derived in the course of this studyDerived in vivo^a

Plasmid	Plasmid specified markers
RM98 (pCU1 + pCU2)	Ap ^r Sm ^r Sp ^r Tc ^r Tra ⁺ Ike ^s PRD1 ^s
pCU1	Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s PRD1 ^s
pCU2	Sm ^r Tc ^r Tra ⁺
pCU4	Ap ^r Sm ^r Sp ^r Tc ^r Tra ⁺ Ike ^s PRD1 ^s
pCU7	Ap ^r Tc ^r Tra ⁺ Ike ^s PRD1 ^s
pCU8	Ap ^r Tra ⁺ Ike ^s PRD1 ^s
pCU9 (pCU8 + pCU2)	Ap ^r Sm ^r Tc ^r Tra ⁺ Ike ^s PRD1 ^s

Derived in vitro^b

Plasmid	Plasmid specified markers
pCU10	Ap ^r Sm ^r Sp ^r
pCU11	Ap ^r Sm ^r Sp ^r
pCU12	Ap ^r Sm ^r Sp ^r
pCU13	Ap ^r Sm ^r Sp ^r
pCU16	Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s PRD1 ^s

^aby conjugation^bby cleaving pCU1 or pCU4 DNA with different restriction endonucleases followed by transformation.

Abbreviations: Ap - ampicillin; Sm - streptomycin; Sp - spectinomycin; Tc - tetracycline; r or s, resistance or sensitivity; Tra - conjugal transfer proficiency; Ike - bacteriophage Ike; PRD1 - bacteriophage PRD1.

Conjugation and analyses of conjugants

The conjugation protocol which was routinely used for N plasmids involves matings on membrane filters. 5 ml of an exponentially growing donor culture in Penassay Broth at 37°C was mixed with 5 ml of an overnight culture of the recipient and immediately filtered through a sterile moist cellulose nitrate filter (0.45 micron porosity, 45 mm diameter). In the conjugation experiments that are referred to as "short-term" matings, the filter bearing the donor and recipient was immediately dropped and diluted into a volume of broth. The dilution thus effected was such that further matings were abolished. It was then incubated for 20 min at 37°C and plated on a medium selective for transconjugants. Such "short-term" matings were of a duration of approximately 30 sec, the time taken for filtration. These "short-term" matings as might be expected, gave rise to a lower frequency of transconjugants than the "long-term" matings. Their purpose was to minimize the simultaneous transfer of more than one plasmid molecule that was potentially present in conjugal donors. For matings that are referred to as "long-term" matings, the filters bearing donors and recipients were placed on the surface of Penassay Agar for 1 hr at 37°C after which they were removed and treated in the same manner as for the "short-term" matings. In addition to this procedure, in some experiments where relevant, standard liquid matings in broth culture were also conducted. Transconjugants that arose from mating experiments were purified and used

further to test for antibiotic-resistance (by replication) conjugative ability (in secondary matings) or sensitivity to the various bacteriophages (by standard platings).

Determining the plating efficiency

The plating efficiency of strains for the different phages was determined by the dilution-plate technique using R top and bottom agar as described by Miller (1972).

Phage lysates

Phage lysates were made by scraping the top agar from plates which showed confluent lysis. R-top agar containing the phage particles was then transferred to a sterile centrifuge tube and spun down at $1000 \times g$ for 10 min (in the case of IKE and PRD1 at $27,000 \times g$ for 30 min). Phage lysates (10^{10} - 10^{11} pfu/ml) were stored at 4°C .

P22-transduction

Transduction by P22 int (integration-deficient) was done according to the procedure described by Ely et al. (1974) and by Schell and Glover (1966). The plasmids to be transduced were transferred by conjugation from E. coli into Salmonella typhimurium LT2-cysD. Overnight cultures of R^+ S. typhimurium LT2 grown in Luria broth (Miller, 1972) were diluted 1:20 in the same prewarmed medium and allowed to reach exponential phase. Since it was observed that RM98⁺ S. typhimurium LT2 restricted P22, a heat pulse at 42°C for 20 min. was given to the exponentially grown cells (Schell

and Glover, 1966). The cells were then infected with P22 phages at a multiplicity of infection of 10. The phages were allowed to preadsorb to the bacteria for 15 min at 37°C. 0.1 ml aliquots were then plated on Penassay agar plates supplemented with Ap or Tc. The remaining phage-bacteria mixture was spun down resuspended in 0.2 ml of L broth and all of it plated. The plates were incubated at 37°C for 24-48 hr.

Isolation of plasmid DNA

The extraction of plasmid DNA was done according to a modification of the Clewell and Helinski procedure (1969). An overnight culture of the desired R⁺ strain was diluted 1:20 into a 1000 ml volume of Penassay broth containing the required antibiotics and grown to the late-exponential phase (approx. 3 hr). The cells were centrifuged at 10,000 x g for 10 min at 0°-5°C in a Sorvall GSA rotor. The pellets were resuspended in 30 ml of cold 25% sucrose in 0.05 M TRIS pH 8.0. 5 ml of freshly prepared lysozyme solution (0.1 g of Sigma 3x crystallized lysozyme in 5 ml of 0.25M TRIS pH 8.0) was added and the suspension was kept on ice for 5 min. Then 25 ml of 0.25M EDTA pH 8.0 solution was added and the mixture was maintained at 0°C (swirling occasionally) for another 5-15 min after which lysis was completed by the addition of 50 ml of a detergent mixture (1% Brij 58, 0.4% sodium deoxycholate, 0.0625M EDTA and 0.05M TRIS pH 8.0). After 5-10 min the mixture became viscous and translucent.

The chromosome-membrane complex was pelleted out of the suspension by centrifuging it for 30 min at 2°C in a SS-34 rotor at 35,000 x g. The supernatant contained plasmid DNA. At this step the Clewell and Helinski method was modified by the precipitation of DNA with PEG 6000 (polyethylene glycol) as proposed by Humphreys et al. (1975).

To the clear lysate, NaCl was added to a final concentration of 0.5M. Then PEG 6000 was added to achieve a concentration of 10% (w/v). The preparation was kept at 4°C overnight. The next day the suspension was spun down in the GSA rotor (4000 x g for 7-10 min). The pellet was resuspended in 7 ml of TE buffer (0.01M TRIS-HCl, 0.001M EDTA, pH 7.2) to which 8 g of solid CsCl and 1 ml of a 10 mg/ml solution of ethidium-bromide was added. The sample was transferred to a nitrocellulose tube which was then centrifuged in a SW-40 Beckman ultracentrifuge rotor. After 48 hr centrifugation at 270,000 x g at 15°C the lower supercoiled DNA band was removed by puncturing the tube from the side. Ethidium bromide was removed by three successive washes of the sample with CsCl saturated-isopropanol solution. CsCl salt was removed by diluting the sample with equal volume of TE buffer and then the addition of two volumes of cold absolute ethanol. The resulting two phases were slowly mixed. The alcohol-salt solution was kept at -20°C overnight. It was then spun down at 3000 x g for 7 min. The ethanol precipitation step was repeated. The DNA preparations were kept in ethanol at -20°C for long periods. Just before use they were

spun down and resuspended in 100 ul of TE buffer.

Restriction endonuclease digestion and electrophoresis of
plasmid DNA fragments

Plasmid DNA preparations were digested to completion with several different restriction endonucleases used separately or in combinations of two or three. The reaction mixtures for the enzyme reactions were prepared as recommended by New England Biolabs and digestions were done in 30 ul volumes. The reaction was stopped by placing the mixture in a 70°C water bath for 10 min. Electrophoresis of DNA fragments was performed in agarose slab gels (concentration 0.7 to 1.4% in different experiments) as described by Tanaka and Weisblum (1975). The gels were composed of the agarose, 135 ml of distilled water and 15 ml of 10x E buffer (0.4M TRIS, 0.2M sodium acetate, 0.01M EDTA, adjusted to pH 8.0 with glacial acetic acid). After melting the agarose, 75 ul of a 1 mg/ml aqueous solution of ethidium bromide was added, mixed and the gel cast. The dimensions of the gel were 140 x 120 x 3 mm.

The electrophoresis buffer was the 1x E buffer of Tanaka and Weisblum (1975) containing 0.5 ml of the ethidium bromide solution per litre. Prior to loading, samples were mixed with 5 ul of a solution containing 50% glycerol and 0.25% bromophenol blue. Vertical gel electrophoresis was conducted at 20 V for 14 hr at room temperature. A HindIII digest of bacteriophage lambda DNA was run simultaneously in

one of the wells, the HindIII fragments of this DNA providing molecular weight standards for the fragments of interest (Murray and Murray, 1975).

Gels were photographed under long-wave (approximately 360 nm) ultraviolet light with a Polaroid MP4 vertical copying camera using a #8 red and yellow filter attached to the lens.

Generation of deletion mutants

Plasmid DNA (approximately 2 ug) was digested separately with different restrictive endonucleases. The reactions were allowed to occur for 3 hr and then the mixtures were heated for 10 min at 65°C. After cooling, the restriction fragments were added to competent cells of either E. coli strain 490 or C600. Transformation was conducted as described by Cohen et al. (1972) and the cells were plated after incubating them for 2 hr at 37°C to allow for phenotypic expression. Transformants were selected on Penassay Agar plates supplemented with either Ap (30 ug/ml), Sp (50 ug/ml) or Sm (50 ug/ml).

Screening for plasmid-associated phenotypes

Growth from each transformant colony was transferred to 0.5 ml of Penassay broth in a microculture container. The containers were placed at 37°C for 4-5 hr. After this period, each bacterial culture was spotted with a multipoint inoculator on dry Penassay agar plates containing antibiotics (Ap, Sp or Sm). To determine whether transformants were

conjugation-proficient, they were spotted on Penassay agar containing Sp (50 ug/ml) and Km (50 ug/ml) which had been spread previously with 10^8 cells of the kanamycin-resistant recipient CR34/pCR1. As two controls, selective plates which were not spread with the recipient strain or spread and spotted with the pCU1⁺ parental strain were used.

To determine the sensitivity of strains to bacteriophage IKE, selected transformant colonies were grown to exponential phase (approx. 5×10^8 cells/ml). 0.2 ml of each culture was spread on freshly made Penassay agar plates. The plates were dried for 40 min and a drop of high titer phage (approx. 10^{11} pfu/ml) was spotted. The presence of a clear zone after 24 hr of incubation at 37°C was taken as sensitivity to phage IKE.

Electron microscopy of plasmid DNA

Samples of plasmid DNA isolated from various strains were prepared for electron microscopy by adding 5 ul of TE buffer containing 0.5 to 1.0 ug of DNA to 0.9 ml of a solution of 50% formamide, 0.1M NaCl, 0.008M Na₂EDTA and 0.1M NaHCO₃, pH 8.5. The samples were spread with cytochrome C (100 ug/ml) on a double distilled water hypophase and picked up with carbon coated formvar films supported by 300 mesh copper grids. The grids were dried by dipping them for 30 sec in absolute ethanol and then shadowed without staining with uranium oxide (Fiandt et al., 1971; Westmoreland et al., 1969). After shadowing, the specimens were examined in a

Siemens 1A electron microscope at a magnification of 5000X. The contour lengths of DNA molecules were measured by projecting a photographic negative through an enlarger to secure approximately X10 magnification and tracing the molecules. pML2 plasmid DNA (8.8×10^6 dal., Hershfield et al., 1974; M. Flandt, personal communication) was included in the preparation and served as an internal length standard.

To detect and visualize 'inverted-repeat loop' structures, a sample containing plasmid DNA was heated to 80°C for 2 min. It was then allowed to reanneal for 2.5 hr at room temperature. The plasmid DNA was then spread for electron microscopy.

Radioactive labelling of plasmid DNA

Incorporation of (α 32 P)dATP into plasmid DNA was performed as suggested by Kelly et al. (1970). The reaction mixture contained 10 μ l of 10x salt solution (50 mM TRIS-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM β mercaptoethanol and 50 μ g/ml BSA), 4-5 μ l of DNA (2 μ g) 5 μ l of (α 32 P)dATP; (New England, specific activity 0.1 mCi in 0.07 ml H_2O) 1 μ l of 10^{-7} g/ml DNAase I (Worthington, freshly diluted from 1 μ g/ml stock solution in distilled H_2O), 1 μ l of DNA Polymerase I (Grade I, Boehringer-Mannheim) and 5 μ l of dNTP (180 pMole of each dCTP, dTTP and dGTP prepared as a combined stock solution in H_2O , each at 1.0 mM). Distilled H_2O was added to achieve the final volume of 100 μ l. The reaction was conducted for 17 hr at 15°C, and then was stopped by adding 10 μ l of 10x

concentrated solution of 0.5% sodium dodecyl sulfate (SDS), 0.01M EDTA, pH 8.4. Unincorporated dNTP's were removed from (α 32 P)dATP labelled plasmid DNA on Sephadex G-50 column (7 cm x 0.6 cm). The Sephadex G-50 was presoaked overnight in the elution buffer (10 mM HEPES buffer; Sigma and 0.01% SDS). Three drop fractions were collected from the column and counted in the H³ channel of a Beckman Scintillation Counter (model L3-230). The counts were plotted and the fractions corresponding to the fast sedimenting peak were pooled and used for the hybridization.

DNA hybridization in liquid

The procedures used for hybridization in the presence of formamide with and without competitor DNA were as described by Kourilsky et al. (1971, 1974). Filter membranes, (0.45 micron porosity, 45 mm diameter, presoaked in 2x SSC solution (1x SSC solution contains 0.15M NaCl; 0.015M sodium citrate; pH 7.5) and washed with 6x SSC were loaded with alkali denatured plasmid DNA in 6x SSC solution. Such DNA-loaded filters were dried in vacuo at 70°C for 2-3 hr. Dried filters were stored in a dessicator over CaCl₂ at 4°C. Blank filters, which served as controls, were subjected to the same treatment except for DNA loading. Before hybridization (α - 32 P)dATP labelled DNA as well as various plasmid competitor DNAs which were used, were fragmented and denatured by boiling for 7 min in 0.5M NaOH (Kourilsky et al., 1971). Subsequently, denatured labelled DNA and different amounts of competitor DNAs were mixed in 4x SSC solution and incubated overnight

at 66°C. Following the prehybridization step the samples were removed from 66°C incubator and formamide (99% Matheson Coleman and Bell Manufacturing Chemists) was added to obtain a final concentration of 40%. 5 mm microfilters were punched out of the "DNA loaded filter" and out of the "blank filter"; and placed in vials. The microfilter loaded with DNA and the blank microfilter were incubated in the same vial. The hybridization mixtures were incubated at 45°C for 3 days with shaking. After 3 days, microfilters were washed 3x with 2x SSC-40% formamide solution with shaking followed by 3 washes with 2x SSC. They were then dried under the lamp, immersed in the scintillation fluid and counted.

Denaturation and transfer of DNA bands in an electrophoresis gel to a nitrocellulose filter ("Southern Blot")

This was done according to the procedure described by Southern (1975) and modified by Wahl *et al.* (1979). A vertical slab gel of 0.7% agarose was photographed as described and exposed to UV light for approximately 10 min to ensure sufficient breakage of the DNA strands for their efficient diffusion from the gel. Subsequently, the gel was immersed in 500 ml of a solution containing 0.4M NaOH and 0.8M NaCl. The denaturation of DNA bands in the gel was allowed to occur with gentle agitation for 30 min. After this time the "denaturation solution" was decanted and 500 ml of neutralizing buffer (0.5M TRIS HCl; 1.5M NaCl, pH 7.5) was added to the gel. The gel was kept in this buffer for 30 min. Following

this step, the DNA bands were transferred from the gel to a nitrocellulose filter by the method of Southern (1975) with 20x SSC (1.5M NaCl, 0.15M Na citrate, pH 7.5) overnight. The next day the outline of the gel was traced with a pencil on the nitrocellulose filter. The gel was then rephotographed to ensure the extent of DNA transfer, and the filter was washed in 3x SSC. The filter was rubbed with a gloved hand to remove any residual agarose particles which would give rise to a high background noise upon autoradiography. In the next step the filter was air-dried for 1 hr and then at 80°C in vacuo for 2 hr to permanently bind the single strands of DNA to the nitrocellulose filter.

Prehybridization and DNA-DNA filter hybridization.

These two steps were done according to the procedure of Wahl et al. (1979). The filter paper was placed in 10 ml of 50% formamide (99% A.C.S. grade, Matheson, Coleman and Bell), 5x SSC, 5x Denhardt's reagent (1x Denhardt's reagent contains 0.02% w/v each of bovine serum albumin, polyvinyl pyrrolidone, and ficol), 50 mM sodium phosphate buffer (pH 6.5), 1% glycine and 250-500 ug/ml alkali denatured salmon sperm DNA (Sigma) in a polyethylene bag. The bag containing the filter was incubated at 42°C for 5-12 hr. After the prehybridization step, the bag was opened, the liquid extruded and 9 ml of a solution containing 50% formamide, 5x SSC, 1x Denhardt's reagent, 20 mM sodium phosphate buffer (pH 6.5) and 100 ug/ml denatured salmon sperm DNA, and 10% freshly

made sodium dextran sulfate, was added to the bag. One ml of the mixture described above, was heated to 65°C for 2 min and the heat-denatured (100°C for 5 min) probe was added to the hybridization buffer. The mixture containing the probe was swirled and added to the polyethylene bag. The hybridization was conducted at 42°C overnight. The following day, the filter was removed from the bag and washed with three 250 ml portions of 2x SSC, 0.1% sodium dodecyl sulfate for 5 min each at room temperature, then with two 250 ml portions of 0.1x SSC, 0.1% sodium dodecyl sulfate at 45°C for a total of 30 min. The radioactivity present in an aliquot of each washing was monitored in a scintillation counter.

Autoradiography

The filter was brought in contact with a sheet of Kodak XR-1 X-ray film loaded in a cassette (Du Pont Cornex Hi-Plus) and exposed for one to seven days. Exposures were done at -20°C to reduce background noise on the film.

RESULTS

7

Within Enterobacteriaceae, the diverse R plasmids that are encountered are classified conveniently into Incompatibility groups (Inc groups — Datta, 1975, 1977). Inc N constitutes one such group. Members of this group occur frequently throughout the world (Hedges et al., 1973; Anderson, 1977; Chabbert et al., 1979). They determine a very efficient conjugation system which is distinct from the intensively-studied F system of conjugation (Willetts and Skurray, 1980) but concerning which there is very little information at the present time. Inc N group plasmids are known to confer on their hosts sensitivity to the filamentous DNA bacteriophage IKE (Khatoon et al., 1972). The initial strain which served as a host for the isolation of this phage was a hospital isolate of Salmonella carrying an N group plasmid referred to as RM98. During several studies conducted in different laboratories, plasmid markers from the original strain have been transferred to various other strains by conjugation or transduction on the general assumption that the original plasmid was now present in all derived strains. Some recent observations on RM98⁺ strains (Khatoon, 1976; Iyer and James, 1978; Grant et al., 1978; Tardif and Grant, 1980) raised the possibility that the plasmid composition of different strains considered to be RM98⁺ on the basis of their genetic markers may in fact be different from one another. An examination of this question was important because a strain referred to as RM98⁺ is held and made available by the Plasmid Reference Center as a prototype of an IKE-

sensitive strain. During the course of this investigation it was observed that while some RM98⁺ strains show genetic instability and segregate plasmid-linked markers at high frequency, others seemed to be more stable. In order to understand the basis of such behavior, these RM98⁺ strains and their plasmids were first studied.

I. Genetic evidence indicating that some RM98⁺ strains carry a plasmid aggregate

a) Evidence for the segregation of plasmids following conjugation

Strains carrying the "plasmid RM98" have the following plasmid-specified phenotypes: resistance to ampicillin (Ap^r); spectinomycin (Sp^r), streptomycin (Sm^r), tetracycline (Tc^r); conjugal transfer proficiency (Tra⁺); and sensitivity to bacteriophages IKE and PRD1 (IKE^s, PRD1^s). The C08/RM98⁺ strain (strain A, Table 5 - see this table for the origin of this and related strains) and other strains closely related to this strain were used as donors in matings with several different R⁻ recipient cells. For simplicity, this group of closely related strains is referred as strain A through G (see Table 5). Following the observation that the N conjugation system is much more efficient on solid surfaces than in liquid media (Dennison and Baumberg, 1975) all matings were conducted on filter membranes as described in Materials and Methods. To minimize secondary matings on filters that this procedure might engender, the mating period was minimized to 30 sec in most experiments and varied in others. The type

Table 5

Strains used as donors of their respective plasmids in conjugation experiments

Strain Designation	Origin	Phenotype	
		Chromosome-specified	Plasmid-specified
A	Conjugal transfer of plasmids from wild-type R ⁻ isolate to C08 followed by selection for an Apr ^r Spr ^r Tcr transconjugant	as for C08 (see Table 3)	Ap ^r Sm ^r Sp ^r Tcr Tra ⁺ Ike ^s
B	As for A but selection for an Apr ^r Spr ^r Tcs transconjugant	as for C08	Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s
C	As for A but selection for an Apr ^r Sps ^r Tcr transconjugant	as for C08	Sm ^r Tcr Tra ⁺
D	Conjugal transfer of plasmids from A to J53 followed by selection for an Apr ^r Spr ^r Tcr transconjugant and maintenance of the resulting strain	as for J53 (see Table 3)	Ap ^r Sm ^r Sp ^r Tcr Tra ⁺ Ike ^s
E	As for A but selection for an Apr ^r Sps ^r Tcr transconjugant	as for C08	Ap ^r Tcr Tra ⁺ Ike ^s
F	As for A but selection for an Apr ^r Sps ^r Tcs transconjugant	as for C08	Ap ^r Tra ⁺ Ike ^s
G	As for A but selection for an Apr ^r Smr ^r Sps ^r Tcr transconjugant	as for C08	Ap ^r Sm ^r Tcr Tra ⁺ Ike ^s

Abbreviations: Ap, ampicillin; Sm, streptomycin; Sp, spectinomycin; Tc, tetracycline; Ike, bacteriophage Ike; Tra⁺, conjugal transfer proficiency; r or s, resistance or sensitivity.

of segregants that arose and their frequency following matings with the RM98⁺ donor and R⁻ recipient are shown in Table 6. Results of this general nature have been obtained repeatedly with several donors and recipients in addition to those shown in Table 6. The primary selection was for Ap^r and separately for Tc^r. The transconjugants obtained were then further tested for other markers by replication. The results indicate the following: a) the frequency of Tc^r transconjugants was consistently lower than the frequency of Ap^r transconjugants (Table 6, donor A); this was independent of the recipient strain used. b) Frequently, transconjugants selected for Ap^r were no longer Tc^r. This occurred both in long-term and short-term matings. c) Frequently, transconjugants selected for Tc^r showed a loss of Ap^r; such a loss was more pronounced in short-term matings than in long-term matings. Analysis of transconjugants by replica-plating, consistently revealed two types of segregants: an Ap^rSm^rSp^rTc^sTra⁺IKe^s type and an Ap^sSm^sSp^sTc^rTra⁺IKe^r type, the frequency of the first being substantially greater than that of the second. Fig. 1 shows diagrammatically the types of conjugational segregants that arose from RM98. The differences observed in the frequency of Ap^rTc^s and Ap^sTc^r, transconjugants and between short-term and long-term matings first raised the possibility that the Ap^r and Tc^r phenotypes were specified by two different conjugative R plasmids. Furthermore, the conjugative transfer of the Ap^rSm^rSp^rTra⁺IKe^s plasmid was more efficient than that of the second Ap^sSm^rSp^sTc^rTra⁺IKe^r plasmid. The Ap^r

FIGURE 1. Conjugal segregation of plasmids from RM98⁺ strains. For simplicity, only Ap^r, Tc^r, Tra⁺ and IKe^s markers included in the diagram.

$Sm^r Sp^r Tc^s Tra^+ Ike^s$ segregant is referred as pCU1 and the Ap^s $Sm^r Sp^s Tc^r Tra^+ Ike^r$ segregant as pCU2 (Table 6). Upon secondary conjugation experiments (Table 6, donors B and C) the markers associated with these plasmids do not segregate any further.

b) Transduction of "RM98 plasmid"-associated phenotypes by *Salmonella typhimurium* phage P22

A $RM98^+$ *S. typhimurium* strain was constructed by conjugation and verified to be $RM98^+$ by the criteria described in (a). Phage P22 was then used to transduce Ap^r and Tc^r separately from the resulting strain (Schell and Glover, 1966; Ely *et al.*, 1974). The frequency of Ap^r transductants was 2×10^{-4} per phage particle and was much higher than the frequency of Tc^r transductants which was 2×10^{-10} per phage particle. The $Ap^r Tc^s$ transductants were also Sm^r and Sp^r . This type of transductional segregant was proficient in bacterial matings with R^- recipients, and conferred sensitivity to the bacteriophage *Ike*. In contrast, the second type of transductional-derivative $Ap^s Tc^r$, could no longer serve as a donor in bacterial matings, and was resistant to *Ike*. These results suggested that while pCU1 associated phenotypes ($Ap^r Sm^r Sp^r Tra^+ Ike^s$) could all be transduced by the same phage particle, the phenotypes linked with pCU2 ($Tc^r Sm^r Tra^+$) could not. Since the best available information (Susskind and Botstein, 1978) indicates that P22 cannot package more than one continuous length of DNA, these results imply that the pCU1-associated phenotypes reside on a single molecule with an upper size limit of approximately,

Table 6

Types of conjugants resulting from the use of different donors in short and long term matings*

Conjugal donor	Time of mating	Transconjugants per 100 donors as selected on		Percent** of tested Apr transconjugants that were:			Percent of tested Tcr transconjugants that were:			Plasmid(s) inferred to be present in the donor and the phenotypes conferred by it			
		Ap	Tc	Sm ^r Spr	Tcr	Ikes	Tra ⁺	Apr	Sm ^r Spr		Ikes	Tra ⁺	
A	30 sec	10 ⁻³	10 ⁻⁵	100	100	1	100	100	6	100	6	100	pCU1 (Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s) pCU2 (Sm ^r Tc ^r Tra ⁺ Ike ^r)
B	30 sec	10 ⁻³	<10 ⁻⁷	100	100	0	100	100	0	100	0	100	pCU1 (Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s) pCU2 (Tc ^r Sm ^r Tra ⁺ Ike ^r)
C	30 sec	<10 ⁻⁷	10 ⁻⁵						0	100	0	100	
A	1 hr	40	10 ⁻¹	100	100	10	100	100	80	100	80	100	pCU1 (Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s) pCU2 (Tc ^r Sm ^r Tra ⁺ Ike ^r)
B	1 hr	38	<10 ⁻⁷	100	100	0	100	100	0	100	0	100	pCU1 (Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s) pCU2 (Tc ^r Sm ^r Tra ⁺ Ike ^r)
C	1 hr	<10 ⁻⁷	10 ⁻¹						0	100	0	100	

*Results of the type shown in this table have been obtained with several recipients. The precise number of transconjugants varied about 5 fold with different E. coli recipients. When used in a secondary conjugant experiment, the primary transconjugants continued to segregate in the same manner or not to segregate depending on whether they carried one of two conjugative plasmids (i.e., pCU1 or pCU2).

**Based on replicating approximately 200 colonies.

27 Md, the molecular weight of DNA in the virus P22 (Rhoades et al., 1968). Using the same type of reasoning Tc^r was inferred to be present on a larger molecule (>27 Md). In addition to these two types of transductants a third type was obtained at a low frequency (2×10^{-7}). This transductant was Ap^r , Sm^r , Sp^r , Tc^r , Tra^+ and IKe^s . Unlike the original $RM98^+$ strain, when this type of transductant was used in a bacterial mating there was no segregation of plasmid markers. Subsequent sections will provide some information about the origin of this type of transductant.

c) Evidence that pCU1 and pCU2 specify different conjugative systems

At the present time and among the Enterobacteriaceae, four independent conjugative systems, the systems F, I, N and P have been recognized on the basis of the isolation of bacteriophages specific for each system. As indicated in the Introduction, the plasmid(s) called RM98 was initially recognized as an N conjugative plasmid on the basis of its ability to confer sensitivity to bacteriophage IKe . Strains carrying segregants of this plasmid all of which were transfer-proficient but only some of which were IKe -sensitive were tested for their sensitivity to the known donor-specific phages R17 (specific for F system), If2 (specific for I system), IKe (specific for N system), IKe h (a mutant of IKe whose host range has been reported to extend to P incompatibility group plasmids), PRR1 and Pf3 (specific for P conjugative group

plasmids) and PRD1 (known to infect P, N and W Incompatibility group strains). The results of these tests are shown in Table 7.

They confirm that pCU1 is a plasmid of the conjugative group N. pCU2 did not confer sensitivity to any of the phages tested. This indicated that pCU2 is either a naturally occurring phage-resistant mutant of a plasmid that belongs to a conjugative system already known among the Enterobacteriaceae of that it represents a novel conjugative system. Further experiments would be necessary to address this question. Since the subject of this investigation was the N group of plasmids, it is this plasmid and its derivatives that have formed the subject of further detailed studies in this thesis. In a subsequent section it will be shown that pCU1 and pCU2 share no detectable physical homology. Since conjugative ability is expected to be specified by a group of genes comprising a significant portion of the genome of a conjugative R plasmid (Tanaka et al., 1976; Silver et al., 1977; Barth et al., 1978), this would suggest that pCU1 and pCU2 specify different conjugative systems.

d) Reconstruction of strains similar to the parental RM98⁺

The fact that pCU1 and pCU2 cohabit the same cell in a stable manner indicates that they are intracellularly compatible with one another, i.e. they are in different incompatibility groups. This conclusion was further supported by experiments leading to the reconstruction of a plasmid-aggregate

Table 7

Susceptibility of strains carrying RM98 and its derivatives to bacteriophages Ike, PRD1, PRR1 and Pf1

Strain	Plasmid inferred to be present in strain	Plasmid-specified phenotype (other than phage-sensitivity)	Sensitivity to phage				
			Ikehl	Ike	PRD1	PRR1	If1 Pf3
A	pCU1 + pCU2	Ap ^r Sp ^r Sm ^r Tc ^r Tra ⁺	+	+	+	-	-
B	pCU1	Ap ^r Sp ^r Sm ^r Tc ^s Tra ⁺	+	+	+	-	-
C	pCU2	Ap ^s Sp ^s Sm ^r Tc ^r Tra ⁺	-	-	-	-	-
D	pCU4	Ap ^r Sp ^r Sm ^r Tc ^r Tra ⁺	+	+	+	-	-

which was indistinguishable in behavior from the original RM98⁺ strain. The results of conjugation between a strain carrying pCU1 with one carrying pCU2 are shown in Table 8. In secondary conjugation experiments, such reconstructed strains behaved in the same manner as the original strain carrying the two plasmids.

e) Contour lengths of plasmid DNA molecules extracted from strains carrying RM98, pCU1, or pCU2 plasmids

The evidence obtained from the various genetic experiments presented so far was consistent with the presence of more than one type of conjugative plasmid molecule in the RM98⁺ strain. This was confirmed directly by electronmicroscopic analysis of plasmid DNA from strains harbouring either RM98, pCU1 or pCU2. The length of plasmid DNA molecules extracted from these strains was determined by electronmicroscopy as described under Materials and Methods. Only intact open circular forms of plasmid DNA were used for the measurements. DNA of plasmid pML2 (Hershfield *et al.*, 1974) was included on the same grids and also measured as an internal standard. The distribution of the lengths of plasmid DNA from the pCU1⁺ strain B indicates two length classes (Fig. 2 A and B). The length of the molecules in the minor class is consistent with the possibility that they represent dimers of the molecules in the major class. This is supported by the observed agreement in molecular weight of the plasmid DNA monomer as determined by electronmicroscopy (26 ± 0.5 Md)

Table 8

Reconstruction of strains similar to the wild-type RM98⁺ strain from its two segregants

Recipient	Plasmid-specified phenotype of recipient	Donor	Plasmid-specified phenotype of donor	Number of Ap ^r or Tc ^r transconjugants per 100 donors
WDOO	no plasmid	C08/pCU2	Ap ^s Sm ^r Sp ^s Tc ^r Ike ^r Tra ⁺	1 x 10 ⁻¹ (Tc ^r)
WDOO	no plasmid	C08/pCU1	Ap ^r Sm ^r Sp ^r Tc ^s Ike ^s Tra ⁺	3 (Ap ^r)
WDOO/pCU1	Ap ^r Sm ^r Sp ^r Tc ^s Ike ^s Tra ⁺	C08/pCU2	Ap ^s Sm ^r Sp ^s Tc ^r Ike ^r Tra ⁺	1 x 10 ⁻¹ (Tc ^r)
WDOO/pCU2	Ap ^s Sm ^r Sp ^s Tc ^r Ike ^r Tra ⁺	C08/pCU1	Ap ^r Sm ^r Sp ^r Tc ^s Ike ^s Tra ⁺	5 (Ap ^r)

On further testing, these transconjugants were found to (a) have the phenotype of the wild-type strain, i.e. Ap^r Sm^r Sp^r Tc^r Ike^s Tra⁺; and (b) when one of each of them was tested in a secondary conjugation they gave the same type and frequency of plasmid-specified segregation patterns as the parental wild-type strain.

FIGURE 2. Length distribution of circular plasmid DNA molecules isolated from strains carrying:

A) RM98 - molecular weights 26.0 ± 0.5 Md; 52.0 ± 1.0 Md and 79.0 ± 2.0 Md.

B) pCU1 - molecular weights 26.0 ± 0.5 Md; 52.0 ± 1.0 Md.

C) pCU2 - molecular weight 79.0 ± 2.0 Md.

Molecular weights were calculated on the assumption that 1 μ m of DNA equals 2.07×10^6 daltons (Lang, 1970).

2

FIGURE 3. 0.7% agarose gel electrophoresis of total plasmid DNA isolated from strains carrying: A - RM98; B - pCU1; C - pCU2. Plasmid DNA was cleaved separately with two different endonucleases. The photograph on the left displays the PvuI restriction endonuclease digest of the plasmids. The photograph on the right shows the HpaI generated fragments. Beside each photograph, a graphical indication of all the fragments that were observed is included. For technical reasons discussed in the text not all of the pCU2 fragments are displayed in the digest of RM98 DNA. The two upper bands which appear in the digest of pCU1 (B) with PvuI enzyme are artifacts and were not observed in other cleavages using the same enzyme.

A B C

A B C

pcu1 pcu2
pcu1 pcu2

HpaI

PvuI

and as will be shown in a subsequent section by summation of the molecular weight of the fragments generated from the plasmid by digestion with restriction endonucleases (26.3 ± 0.3 Md). The plasmid DNA in the pCU2⁺ strain C (Fig. 2A and C) is substantially larger than pCU1 and has a molecular weight of approximately 79 ± 2 Md (Fig. 2C). This confirms that it is a different plasmid. As expected, RM98⁺ strain A is seen to contain both of these plasmids (Fig. 2A).

f) Restriction endonuclease cleavage patterns of plasmid DNA extracted from strains inferred to carry the segregant plasmids or their aggregate

Plasmids extracted from strains carrying RM98 (strain A); pCU1 (strain B) or pCU2 (strain C) were each cleaved separately (to completion) with several different restriction endonucleases (BamHI, BglIII, EcoRI, HindIII, HpaI, PvuI, SalI, XhoI and pairs of these enzymes), and electrophoresed on agarose slab gels as described under Materials and Methods. Fig. 3 displays examples involving two enzymes (PvuI and HpaI). The patterns of the plasmid DNA extracted from strain A are consistent with a conclusion that this DNA is a mixture of pCU1 and pCU2 plasmid DNA molecules. All bands present in B are present in A and the pronounced bands in C are also present in A. The absence in A of some of the faint bands seen in C is probably because the plasmid DNA preparation from the strain carrying the aggregate of the two plasmids (strain A) carries more copies of pCU1 DNA than of pCU2.

This is generally evident from the relative intensities of bands emerging from pCU1 and pCU2 for both restriction enzymes regardless of whether the strains carry either one or both plasmids. It was shown that pCU2 plasmid DNA molecules are longer than pCU1. It may therefore be more susceptible to nicking than is pCU1 during DNA extraction and purification so that a smaller proportion of it is recovered. Alternatively, the number of pCU2 plasmid DNA molecules within cells may be less than that of pCU1. This was not studied further as it was not directly relevant to our objective. Fig. 3 indicates that pCU1 and pCU2 DNA have distinguishable band patterns for two enzymes and similar results were obtained with the other enzymes used. pCU1 DNA does not have a cleavage site for XhoI. pCU2 on the other hand was cleaved into seven fragments by this enzyme. Taken with the evidence from the conjugation experiments (Table 5), these results indicate that strain A carries an aggregate of two different and largely unrelated plasmids.

II. Spontaneous derivatives arising from strains carrying the RM98 plasmid aggregate

a) Comparison of plasmid pCU4 with the original RM98

The cohabiting plasmids pCU1 and pCU2 are inferred to be physically unrelated and usually stably inherited. Rarely, however, derivatives can arise following conjugation which do not behave like the original RM98⁺ strain carrying the plasmid aggregate. The properties and behavior of strain D which carries a plasmid designated as pCU4 (Table 10,

strain D) are of special interest. This strain which has all of the RM98-specified phenotypes displayed a different segregation pattern of plasmid markers upon conjugation. In bacterial matings, strain D (pCU4⁺) did not behave like strain A (RM98⁺). Only one class of transconjugant similar to the donor in its plasmid specified phenotype was observed (Table 10, strains A and D).

The antibiotic-resistance phenotype (Ap^r Sm^r Sp^r Tc^r) of strain D, its sensitivity to bacteriophages IKE and PRD1, the single type of transconjugant it gave rise to in mating reactions and the frequency of such transconjugants relative to those obtained with donors carrying either pCU1 or pCU2 (Table 9) all suggested that it carried a single plasmid (pCU4) which although conferring resistance to tetracycline, was similar to pCU1. To confirm and extend this observation, plasmid DNA was purified from strain D, cleaved with different restriction endonucleases, electrophoresed, and the resulting patterns compared to those of plasmid pCU1 (Fig. 4). The length of pCU4 plasmid DNA molecules was also compared to those of pCU1 by electronmicroscopy (Fig. 6). Both Figures 4 and 6 indicate that the length of pCU4 DNA is greater than that of pCU1. Fig. 4 shows that for any one endonuclease, there are some fragments of pCU4 with a mobility similar to those of pCU1 and others that are unique to pCU4 (Fig. 4). The increment in length of pCU4 over pCU1 as determined by the summation of the molecular weights of their fragments (Table 9) is in reasonable agreement with the molecular weight

FIGURE 4. Comparison of the agarose gel electrophoretic patterns of the DNA of pCU4 and that of pCU1 after cleavage with various restriction endonucleases. For each enzyme, the left panels shows the pattern generated from pCU1 and the right panel the pattern generated from pCU4.

A) - pCU1 and pCU4 cut with BglII

B) - pCU1 and pCU4 cut with HpaI

C) - pCU1 and pCU4 cut with PstI

D) - pCU1 and pCU4 cut with SalI

E) - pCU1 and pCU4 cut with EcoRI

F) - pCU1 and pCU4 cut with BamHI

Molecular weight estimates of the fragment generated by these restriction endonucleases are indicated in table 9. Bacteriophage λ DNA cut with HindIII was always included in each gel and served as a molecular weight standard (Murray and Murray, 1975).

A

B

C

D

E

F

difference that was determined by electronmicroscopy. It was concluded that strain D carries only a single plasmid pCU4 and that this was an N group plasmid derived from pCU1 and a region of approximately 8.0 ± 0.7 Md conferring Tc^R contributed by pCU2 from the parental strain A.

b) Properties of other spontaneous derivatives of RM98

Strains E ($pCU7^+$), F ($pCU8^+$) and G ($pCU9^+$) were detected as strains that had lost respectively the markers Sm^R and Sp^R , Sm^R Sp^R and Tc^R , and Sp^R (Table 4, and also Fig. 5). As shown in Table 10, all plasmid-associated markers were transferred together from strains E and F and no segregation of plasmid-associated markers was detected. The conjugal behavior of these strains, their sensitivity to bacteriophage IKE and their inability to yield segregants upon conjugation (Table 10) all suggested that they had lost the plasmid pCU2 and retained some derivative of pCU1. The plasmids in these strains were inferred to be different derivatives of the parental N group plasmid pCU1 and designated pCU7 and pCU8. Plasmids pCU4 and pCU7 are Tc^R and transmit this resistance as part of the N group plasmid (Table 10). The inference would be that in these two cases, Tc^R has been added from pCU2 (which is no longer present) to the N group plasmid pCU1 by some recombination reaction between the two plasmids. pCU8, like pCU7, has lost Sm^R and Sp^R , genes initially associated with pCU1 and has also lost all the pCU2 markers. However, unlike pCU7, it has not acquired the Tc^R phenotype. This

Table 10
Transmission of plasmid markers following conjugation

Conjugal donor	Type of mating***	Transconjugants per 100 donors*	Percent** of tested Ap ^r transconjugants that were:	Percent of tested Tc ^r transconjugants that were:	Plasmid(s) inferred to be present in the donor and the phenotypes conferred by it
		Ap ^r	Sm ^r Sp ^r Tc ^r Ike ^s Tra ⁺	Ap ^r Sm ^r Sp ^r Ike ^s Tra ⁺	
A	short term	10 ⁻³	100 100 1 100 100	6 6 6 6 100	pCU1 (Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s)
	long term	40	0.2 100 100 20 100 100	80 100 80 80 100	pCU2 (Sm ^r Tc ^r Tra ⁺)
B	short term	10 ⁻³	<10 ⁻⁷ 100 100 0 100 100		pCU1 (Ap ^r Sm ^r Sp ^r Tra ⁺ Ike ^s)
	long term	38	<10 ⁻⁷ 100 100 0 100 100		
C	short term	<10 ⁻⁷	10 ⁻⁵	0 100 0 0 100	pCU2 (Tc ^r Sm ^r Tra ⁺)
	long term	<10 ⁻⁷	0.1	0 100 0 0 100	
D	short term	10 ⁻³	100 100 100 100 100 100	100 100 100 100 100	pCU4 (Ap ^r Sm ^r Sp ^r Tc ^r Tra ⁺ Ike ^s)
	long term	25	7 100 100 100 100 100	100 100 100 100 100	
E	short term	10 ⁻²	10 ⁻³ 0 0 100 100 100 100	0 0 100 100 100	pCU7 (Ap ^r Tc ^r Tra ⁺ Ike ^s)
	long term	5	3 0 0 100 100 100 100	0 0 100 100 100	
F	short term	10 ⁻⁴	<10 ⁻⁷ 0 0 0 100 100		pCU8 (Ap ^r Tra ⁺ Ike ^s)
	long term	8	<10 ⁻⁷ 0 0 0 100 100		
G	short term	10 ⁻⁴	10 ⁻⁵ 0 0 0 100 100 100 100	0 100 100 100 100	pCU8 (Ap ^r Tra ⁺ Ike ^s)
	long term	5	0.5 6 0 6 100 100 100 100	0 100 100 100 100	pCU2 (Sm ^r Tc ^r Tra ⁺)

* Results of the type shown in this table have been obtained with several recipients. The precise number of transconjugants varied about 5 fold with different *E. coli* recipients. When used in a secondary conjugation experiment, the primary transconjugants continued to segregate in the same manner or not to segregate depending on whether they carried one or two conjugative plasmids (i.e., pCU1 or pCU2).

**Based on replicating approximately 200 colonies.

***Matings were on membrane filters for either: short-term 30 sec at 37°C, or long-term 1 hr at 37°C.

FIGURE 5. Origin of plasmids arising upon conjugation from RM98⁺ strains.

RM98

(Ap^r Sm^r Sp^r Tc^r Tra⁺ Ike^s)

— frequent event

--- rare event

PCU1

Ap^r Sm^r Sp^r Tra⁺ Ike^s

PCU8-like

Ap^r Tra⁺ Ike^s

PCU2

Tc^r Sm^r Tra⁺
IKer^r

PCU8

Ap^r Tra⁺ Ike^s

PCU9

Ap^r Sm^r Tc^r Tra⁺ Ike^s

PCU4

Ap^r Sm^r Sp^r Tc^r Tra⁺ Ike^s

PCU7

Ap^r Tc^r Tra⁺ Ike^s

suggests that the loss of markers from pCU1 can occur without any observable interaction with pCU2 (i.e. the addition of Tc^r from pCU2 to pCU1). The antibiotic resistance and conjugal behavior of pCU9 (in strain G) is consistent with this suggestion. This strain has lost the pCU1-associated Sp^r marker but retains Tc^r and following a conjugation reaction, it segregates in a manner suggesting that it retains pCU2 and the altered derivative of pCU1 (Table 10). It thus seems that the loss of antibiotic-resistance markers from pCU1 is not necessarily coupled to either an observable interaction with pCU2 or the loss of the latter plasmid. To support the above statements and to obtain information on the physical structure of all of these spontaneous derivatives arising from RM98, purified plasmid DNA from pCU4⁺, pCU7⁺, pCU8⁺ and pCU9⁺ strains were cleaved with several different restriction endonucleases (BamHI, EcoRI, HindIII and PvuI and electrophoresed. Fig. 6 displays the resulting electrophoretic pattern for plasmids from strain D (pCU4⁺), E (pCU7⁺) and F (pCU8⁺). From their sensitivity to IKe and PRD1 and their behavior as conjugal donors (Table 10) all three strains were inferred to carry only an N group plasmid related to pCU1 and not to carry pCU2. For each enzyme used, the pattern obtained with the parental pCU1 and also with pCU2 DNA for comparison is included. (The extreme left and right panels for each enzyme.) For each of the enzymes used, the plasmids pCU4, pCU7 and pCU8 have some fragments in common with pCU1 and others that are different. This is consistent with the

FIGURE 6. Comparison of the 0.7% agarose gel-electrophoretic patterns of the plasmid DNA extracted from strains carrying pCU1, pCU2, pCU4, pCU7 and pCU8.

- A: BamHI digest of (from left to right) pCU1, pCU4, pCU7, and pCU2.
- B: EcoRI digest of (from left to right) pCU1, pCU4, pCU7, pCU8 and pCU2.
- C: HindIII digest of (from left to right) pCU1, pCU4, pCU7 and pCU2.
- D: PvuI digest of (from left to right) pCU1, pCU4, pCU7, pCU8 and pCU2.

A

B

C

D

inference (from the phenotypes conferred by these plasmids) that they are derivatives of pCU1 that are distinguishable from one another. pCU4 and pCU7, although pCU1-related, transmit Tc^r , a marker originally present in pCU2 and not in pCU1 in the plasmid aggregate. A comparison of the restriction patterns of pCU4 and pCU7 to the parental pCU1 shows that in both cases new bands are acquired together with Tc^r phenotype (see BamHI, EcoRI and PvuI digests, Fig. 6). pCU8 which is a derivative of pCU1 that has irreversibly lost the Sp^r and Sm^r markers yields a DNA restriction pattern consistent with a conclusion that it has suffered only a deletion(s) (Fig. 6). Apart from having an addition of DNA sequences, pCU7 also suffered a deletion (Fig. 6). Additional evidence supporting and extending these observations come from the electronmicroscopic measurements of the contour lengths of pCU1, pCU4, pCU7 and pCU8 plasmid DNA molecules (Fig. 7). pCU4 and pCU7 DNA have molecular weights of 34.0 ± 1.0 Md and 31.5 ± 0.7 Md respectively, compared to 26.0 ± 0.5 Md for pCU1 DNA. On the other hand pCU8 DNA has a molecular weight of 24.0 ± 0.5 Md. These molecular weights are in good agreement with the molecular weights obtained by the summation of fragment sizes generated by PvuI endonuclease (Table 11).

Unlike pCU4, pCU7 and pCU8 the behavior of pCU9 in conjugation experiments (Table 10) indicates that in addition to an altered derivative of pCU1 it also carried pCU2. The electrophoretic banding pattern of enzyme-digested plasmid DNA supports this conclusion (Fig. 8). For each restriction

Table 11

Individual and aggregate molecular weight of fragments of
DNA obtained by complete digestion of different plasmid
DNA's with the restriction endonuclease PvuI

(see also Figs. 2 and 7)

Fragment	pCU1	pCU2	pCU4	pCU7	pCU8
1	12.5	14.8	12.5	12.5	12.5
2	5.7	11.2	7.2	7.2	5.7
3	4.6	10.8	4.6	4.6	4.6
4	1.1	9.1	2.4	2.4	1.0
5	1.0	7.6	2.0	2.0	0.9
6	0.9	6.3	1.6	1.6	0.4
7	0.4	4.6	1.1	1.0	
8		3.0	1.0	0.9	
9		2.9	0.9	0.4	
10		2.8	0.4		
11		2.4			
12		2.1			
13		1.7			
Total	26.2	79.3	33.8	32.6	25.1

The molecular weights of fragments were determined by comparing the relative mobility in the gel of each PvuI fragment to the mobility in the same gel of λ DNA fragments obtained with complete digestion by HindIII (Murray and Murray, 1975).

FIGURE 7. Length distribution of circular plasmid DNA molecules isolated from strains carrying;

A) pCU1 - molecular weight 26.0 ± 0.5 Md

B) pCU4 - molecular weight 34.0 ± 1.0 Md

C) pCU7 - molecular weight 31.5 ± 0.7 Md

D) pCU8 - molecular weight 24.0 ± 0.5 Md

Molecular weights are calculated on the assumption that 1 μ m of DNA equals 2.07×10^6 daltons (Lang, 1970).

FIGURE 8. 0.7% agarose gel electrophoretic pattern of pCU9 plasmid DNA cleaved with BglII, HpaI and PvuI enzymes. The numbers indicate the bands arising from either pCU2 or pCU8 DNA.

Bgl II

Hpa I

Pvu I

endonuclease used, several bands that are characteristic of pCU2 are seen in this preparation. As in the case of RM98 and presumably for the same reason, the less pronounced bands could not be visualized. However, and as in the case of RM98, this plasmid aggregate could be shown to carry pCU2 because it could give rise to a segregant carrying a plasmid that is genetically and physically indistinguishable from pCU2. The bands arising from pCU9 that are not accountable by pCU2 arise from its pCU1-related plasmid component. As may be expected, these bands are more pronounced and their pattern is similar to the pCU1 derivative, pCU8. This confirms that pCU1 can undergo physical as well as genetic alterations in the absence of an observable interaction with pCU2. To further substantiate these results, pCU9 DNA was examined by electron microscopy. Fig. 9 shows that pCU9 DNA contains two types of plasmid molecules. The smaller molecules are comparable in size to pCU8 and as expected the bigger molecules have the same molecular weight as pCU2. (pCU1 has been included in this histogram for comparison.)

c) Competition DNA hybridization experiments

The evidence obtained from the conjugation experiments (Table 10), from the restriction endonuclease cleavage patterns, (Fig. 6) and from the electron microscopic analysis (Fig. 2) indicated that pCU1 and pCU2 are physically and genetically unrelated plasmids. This was confirmed by reciprocal DNA hybridization experiments in which either denatured pCU1 DNA or denatured pCU2 DNA was affixed on filters and the ability

FIGURE 9. Length distribution of circular plasmid DNA molecules isolated from strains carrying:

- A) pCU1 - molecular weight 26.0 ± 0.5 Md
- B) pCU2 - molecular weight 79.0 ± 2.0 Md
- C) pCU8 - molecular weight 24.0 ± 0.5 Md
- D) pCU9 - molecular weights 24.0 ± 0.5 Md and 79.0 ± 8.0 Md.

Molecular weights are calculated on the assumption that 1 μ m of DNA equals 2.07×10^6 daltons (Lang, 1970).

(Dimeric molecules of pCUI or of its derivatives were observed (see Fig.2) but have not been included in this Figure to preserve clarity).

NUMBER OF MOLECULES

LENGTH (μm)

of radioactive homologous denatured fragments to bind to it in the presence of competitor DNA fragments studied. This procedure has been found to be a reliable measure of sequence similarity (Kourilsky et al., 1974). Two other plasmid DNA preparations were also included as competitor DNA in these studies: the plasmid DNA from strain D that had been referred to carry the pCU1-related plasmid carrying the Tc^r region, (pCU4) and the plasmid DNA from strain G that was inferred to carry both a pCU1-related plasmid pCU8 and pCU2, (pCU9). The results of these experiments are shown in Figs. 10A and B. Neither pCU1 DNA nor pCU2 DNA is found to compete with the respective heterologous DNA for hybridization. pCU4 plasmid DNA from strain D competes well with pCU1 DNA but only a little with pCU2 as would be expected from its inferred greater relationship to pCU1. pCU9 (=pCU2 + pCU8) DNA from strain G competes effectively with both pCU1 and pCU2 DNA. This is an expected result for a strain that carries pCU2 and a substantial portion of pCU1.

III. Electron microscopic analysis of pCU1 plasmid and its derivatives

The results of conjugation experiments and restriction endonuclease analyses suggested that pCU1 could undergo genetic and physical alterations, which would include deletions or/and additions of different DNA sequences. These changes were linked to the acquisition or loss of some antibiotic resistance markers (e.g. addition of Tc^r or deletion of Sm^s).

FIGURE 10. Competition hybridization between pCU1 DNA and the DNA of other plasmids.

A. Samples containing 2×10^5 cpm of ^{32}P -labelled denatured pCU1 DNA prepared as described by Kourilsky et al. (1974) and in Materials and Methods were hybridized to 2 ug of denatured pCU1 DNA bound to microfilters. pCU1-pCU1 hybridization was followed in the presence of variable amounts of either pCU2 DNA (from strain C), pCU4 DNA (from strain D), or pCU9 DNA (from strain G) as competitors.

B. Samples containing 1×10^5 cpm of ^{32}P -labelled, denatured pCU2 DNA were hybridized to 2 ug of denatured pCU2 DNA bound to microfilters. pCU2-pCU2 hybridization was followed in the presence of either pCU1, pCU4 or pCU9 DNA as competitors.

and Sp^r markers).

It is well documented that some antibiotic resistance genes reside on discrete genetic units named "transposons" (Kleckner, 1977; Calos and Miller, 1980). Electronmicroscopic analysis of denatured plasmid DNA has revealed that some transposons have characteristic double-stranded stem and single-stranded loop structures. The double-stranded stem of the transposon consists of DNA segments repeated on the same strand of DNA in inverted orientation. These inverted repeats may be close to each other or far apart. During the separation of the DNA double helix by heat or alkali rapid intrastrand annealing of the repeated sequences results in the formation of a single-stranded loop, which contains the duplicated sequences as the double-stranded stalk (Sharp et al., 1973; Cohen, 1976). The inverted repeats of transposable elements have been implicated in a variety of recombination events, involving insertions, deletions and duplications of DNA sequences (Kleckner et al., 1978; 1979). Since pCU1 was observed to give rise to such mutants it was of interest to see whether such stem-loop structures would be formed on denatured pCU1 DNA.

a) Stem-loop structures observed in pCU1 DNA molecules

pCU1 plasmid DNA was heat-denatured allowed to renature as described in Materials and Methods, and single-stranded molecules were examined in the electronmicroscope. Electronmicrographs of the different types of stem-loop structures

observed are shown in Figs. 11, 13, 14 and 15. Fig. 11 illustrates a single-stranded molecule with two such stem-loop structures labelled A and B. Interestingly, the smaller stem-loop structure (B) is within the larger loop of A. It should be pointed out that under the conditions of denaturation and renaturation, in any given pCU1 molecule not all possible stem-loop structures will form. For example, in some molecules the B stem-loop may not form. Provided the stem-loop A was formed, this would then give rise to a structure comprised of the stem of A and a loop the length of which would be the sum of the A loop and the total length of the B stem and loop. This is illustrated diagrammatically in Fig. 12. By examining a large number of pCU1 molecules, it was possible to describe and measure all of several kinds of stem-loop structures that could be formed and seen in pCU1. It was also possible to determine their relative distribution on the plasmid DNA. The information derived from such an analysis is presented diagrammatically in Fig. 12 and Figs. 13 and 14 are representative electronmicrographs of molecules with the different stem-loop structures. As illustrated in these micrographs and the accompanying diagrams, four types of stem-loop structures called A, B, (or A + B), C and D were visualized among the molecules of pCU1 DNA. A single molecule in which all four of these structures were formed was relatively rare. An example is shown in Fig. 15. Table 12 (see also Fig. 15) provides a summary of the morphology and distribution of these stem-loop structures. These

FIGURE 11: Electronmicrograph showing single-stranded molecule of pCU1 with two stem-loop structures (A and B).

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FIGURE 12. Distribution of stem-loop structures observed in pCU1 molecules. The stems and loops of three structures A, C and D are drawn. Structure B is not formed and is shown as a part of structure A. Its inverted repeats and the intervening loop are indicated as B. The numbers represent the distances between stem-loop structures, e.g. the distance between structure A and D is 1.5 ± 0.3 Md in pCU1 molecule. The values are expressed in megadaltons.

B-stem

FIGURE 13. Single strand of pCU1 plasmid DNA showing three stem-loop structures: A+B and C. The distance between structure A and C is 1.5 ± 0.3 Md. In the explanatory drawing the double-stranded regions are drawn with heavier lines.

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FIGURE 14. Different types of inverted repeat-loop structures observed in single-stranded pCU1 molecules. The numbers in brackets designate the number of structures measured. The sizes are expressed in megadaltons.

Structure A (52)	stem - 0.2 ± 0.03 Md
	loop - 3.7 ± 0.4 Md
Structure B (35)	stem - 0.09 ± 0.01 Md
	loop - 0.6 ± 0.1 Md
Structure C (17)	stem - 0.1 ± 0.1 Md
	loop - 1.3 ± 0.3 Md
Structure D (36)	stem - 0.15 ± 0.02 Md
	loop - 2.2 ± 0.3 Md

A

A+B

C

D

FIGURE 15. Electronmicrograph representing a single-stranded molecule of pCU1 in which all four (A, B, C and D) stem-loop structures are formed.

electronmicroscopic studies have thus indicated that pCU1 DNA molecules contain complementary inverted-repeat sequences that can form at least four kinds of stem-loop structures. Since the morphology of each stem-loop structure is constant, within the limits of the number of molecules examined, the inverted repeat sequences must have a constant position on the plasmid DNA. The measurement of distances between the stem-loop structures also supported this conclusion.

b) Location of Tc^r-determinant in pCU1 derivatives

pCU4 and pCU7 have been inferred to be plasmids related to pCU1 although they specify and conjugatively transmit Tc^r (Table 10), a marker originally associated with pCU2 (see Fig. 27 p 119). The acquisition of Tc^r by pCU1 resulted in the addition of a DNA sequence of 8.0 ± 0.7 Md (Table 9 p. 74). Since pCU1 and pCU2 are shown to be unrelated, these observations suggest that the Tc^r determinant in pCU2 is part of an element that was transposed from pCU2 to pCU1. In order to further examine this possibility pCU4 and pCU7 DNAs were denatured and analyzed under the electronmicroscope. In addition to the A, B, C and D stem-loop structures of pCU1 another stem-loop structure was observed in both pCU4 and pCU7. Fig. 16a represents this new stem-loop structure designated as E. The measurements of eleven molecules bearing this stem-loop indicated its size to be 6.7 ± 0.6 Md. In a previous section of Results (Part II, Section 6, Fig. 7 p 83) from a comparison of the contour-lengths of double-stranded

DNA molecules of pCU4 and pCU1, it was inferred that pCU4 was greater than pCU1 by approx. 8.0 Md. A possible explanation for the apparent discrepancy between this value and the value of 6.7 Md for the new stem-loop structure E, is that the latter value is based largely on measuring single-stranded rather than double-stranded regions of DNA. The electron microscopic measurements on single-stranded DNA are likely to underestimate length because of shrinkage and kinkiness of the DNA molecule.

In molecules of denatured pCU7 DNA this new 6.7 Md loop is 1.5 Md away from the stem-loop D of pCU1. Since the relationship of structures A, B, C and D on pCU1 have been determined (Fig. 12), it would appear that the insertion of structure E could occur in either clockwise or anticlockwise direction from structure D (Fig. 12). The insertion of stem-loop E in anticlockwise direction would end up in the loop of structure A (Fig. 12). The finding of eight bigger (9.2 ± 0.6 Md) stem-loop structures in the denatured preparations of pCU7 lent some support to this notion. Fig. 17 diagrammatically represents the relationship of stem-loop structures E and A.

c) Stem-loop structures observed in denatured molecules of pCU8

The initial motivation for the search of stem-loop structures in pCU1 was the observation of a degree of spontaneous genetic instability in this plasmid and the conclusion that this genetic instability could occur independent of any

FIGURE 16. Electronmicrographs of stem-loop structures associated with the Tc^r determinant in pCU4 and pCU7 plasmids.

- a) stem-loop structure E
- b) stem-loop structure A + E

a

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b

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FIGURE 17. Diagrammatical representation of the insertion of stem-loop structure E bearing Tc^r into the pCU1 molecule.

Configuration I

Configuration II

interaction with pCU2 (p 85). Since stem-loop structures have now been observed in pCU1 and characterized and spontaneous derivatives of pCU1 that had lost one or more genetic markers were available, it was possible to ascertain whether loss of these markers was associated with changes in any of the stem-loop structures. pCU8 was a good candidate to examine as it had irreversibly lost its Sm^r and Sp^r markers (p 104) and contour length measurements on its double-stranded DNA had indicated that it had suffered a 2 Md deletion (Table 12, Fig. 7). When examined for stem-loop structures, pCU8 DNA was observed to have the structures A, B and D but not C. Fig. 18 illustrates such a molecule (compare to pCU1, Fig. 14). The size of the missing stem-loop structure C (1.7 Md) is sufficiently similar to the estimated size of the deletion (2.0 Md, see p 103 for a possible explanation of this discrepancy) as to suggest that the entire stem-loop structure and not just a part of it (e.g. one of the inverted repeats) has been deleted. This stem-loop structure C probably contains the Sm^r and Sp^r markers as loss of the markers was correlated with loss of C. As pointed out earlier, transposons frequently have stem-loop structures and are also subject to deletion.

IV. Construction of restriction endonuclease map of pCU1

A physical map of pCU1 DNA that is more detailed than the one secured by electronmicroscopy can be useful in localizing more precisely the components of the stem-loop structures

FIGURE 18. Different kinds of stem-loop structures found on single-stranded molecules of pCU8. Two stem-loop structures (A and D) are shown.

described in the previous section and in mapping the various genetic functions that this plasmid specifies. Restriction-endonuclease mapping was a feasible way of accomplishing this objective especially when coupled with the use of various derivatives of pCU1 which had lost one or more of their genetic functions. This section describes the construction of such a map using several restriction endonucleases.

a) Comparison of restriction endonuclease patterns generated by several enzymes from pCU1 and pCU8 DNA

Fig. 19 compares the electrophoretic patterns of pCU1 and pCU8 DNA after they were each digested completely with the restriction endonucleases BamHI and EcoRI. The numerals on the right of each band that is displayed is the molecular weight of that fragment as calculated using HindIII fragments of lambda DNA as standards when they were run simultaneously in one of the gel slots. The molecular weights used for the lambda DNA fragments were as given by Murray (1975). In these and in subsequent molecular weight determinations, the sizes of the largest fragments (~ 20 Md) must be considered to be approximate as in these gel system used, small differences in the molecular weight of fragments greater than 20 Md are not well resolved. Fig. 19 shows that with BamHI, both pCU1 and pCU8 yield 2 fragments. Fragment 1 (from the top) is of approximately the same size for both plasmids but fragment 2 of pCU8 is shorter than fragment 2 of pCU1 by 2 Md (see also Table 11 and Fig. 7 p 83). This observation

FIGURE 19. Comparison of electrophoretic mobilities of fragments generated by BamHI and EcoRI restriction endonucleases from pCU1 and pCU8 DNA. The numbers designate the molecular weights of restriction endonuclease fragments and the values are expressed in megadaltons. The measurements were done using λ DNA digested with HindIII nuclease (Murray and Murray, 1975).

was consistent with the results of electron microscopy and implied that a deletion within fragment BamHI-2 of pCU1 was sufficient to account for the difference between the DNA of pCU1 and pCU8. It also suggested that the deleted portion contained genetic regions influencing Sm^r and Sp^r . EcoRI also produces two fragments from pCU1 but only one from pCU8. The smaller fragment missing in pCU8 was 1.3 Md in size. This observation was consistent with a tentative conclusion that fragment EcoRI-2 was either contained within or overlapped with the fragment BamHI-2 of pCU1. It was possible to discriminate between these two possibilities by examining the products of complete digestion of pCU1 with mixtures of enzymes.

b) Restriction endonuclease cleavage analyses of pCU1 DNA

The rationale used in this type of analyses is illustrated by the results presented for three enzymes in Figs. 20 and 21 and also in Table 12 (Top). The results in Fig. 20 show that HindIII generated from pCU1 DNA, fragments of approx. 25 Md and 1.4 Md and that BamHI generated fragments of approx. 22.0 and 4.0 Md. With the HindIII-BamHI double digest, approx. 22.0, 3.0, 1 Md and 0.4 Md fragments were obtained (Fig. 21). This implies that the smaller HindIII and BamHI fragments overlap and therefore that only one HindIII cleavage site lies within the 4 Md BamHI fragment. The other HindIII site must therefore lie within the large BamHI fragment. EcoRI also produced two fragments from pCU1 with sizes of approx. 25 and 1.3 Md (fig. 21). The mixture of

Table 12

Molecular weights of fragments generated from pCU1 plasmid DNA by various restriction endonucleases used separately or in combination of two

Restriction endonuclease	Molecular weights of fragments (Md)	Total molecular weight (Md)
HindIII	~ 25.0; 1.4	26.4
BamHI	~ 22.0; 4.0	26.0
HindIII + BamHI	~ 22.0; 3.0; 1.0; 0.4	26.4
EcoRI	~ 25.0; 1.3	26.3
BamHI + EcoRI	22.0; 1.5; 1.3; 1.2	
HindIII + EcoRI	~ 23.0; 1.4; 1.3; 0.5	26.4
HpaI	5.5; 4.8; 4.0; 3.6; 2.3; 1.7 (double band); 1.2; 1.0	25.8
KpnI	19.0; 3.8; 3.0	25.8
PstI	~ 22.0; 1.7; 1.5; 0.2	25.4
BglII	10.5; 3.5; 3.3; 2.9; 2.8; 2.4; 0.8	26.0
PvuI	12.5; 5.7; 4.6; 1.1; 1.0; 0.9; 0.4	26.2
SalI	~ 18.0; 6.8; 0.7; 0.3	26.8
SmaI	15.8; 10.9	26.7
KpnI+ EcoRI	12.9; 6.3; 3.6; 2.9; 1.3	25.8
KpnI+ HindIII	14.8; 4.2; 3.6; 2.9; 1.4	26.9
SalI+ BamHI	14.8; 6.8; 4.0; 0.7; 0.3	26.6

The molecular weight standard used was phage lambda restriction fragments ranging in size from 15.0 to 0.32 Md (Murray and Murray, 1975).

FIGURE 20. Restriction endonuclease cleavage of pCU1 DNA. BamHI, EcoRI and HindIII endonucleases were used (from left to right) to cleave the DNA. The fragments were electrophoresed in 0.7% agarose gels. Above the displays of the gel containing DNA fragments, the graphical illustrations of the cuts made by each enzyme on pCU1 molecule is included. The numbers designate the molecular weights of the DNA fragments.

2

FIGURE 21. Double digests of pCU1 DNA (from left to right) by BamHI + EcoRI, BamHI + HindIII, and EcoRI + HindIII. The circles above the gels represent the graphical explanation of the cuts made by the combination of two enzymes in the pCU1 molecule. In the double digest using BamHI - HindIII endonucleases, the small fragment has been eluted out from this 0.7% gel.

BamHI and EcoRI produced fragments of the size approx. 22.0, 1.5, 1.3, and 1.2 Md respectively (Fig. 21 p 107). The latter result is consistent with an explanation that the 4.0 Md BamHI fragment contains within it two cleavage sites for EcoRI. This explanation was also supported by the observation (Fig. 19 p 103) that as a consequence of the deletion generating pCU8 from pCU1, the 1.3 Md EcoRI fragment was lost and that this loss was correlated with a reduction in the size of the 4.0 Md BamHI fragment. In order to position the EcoRI and HindIII cleavage sites in relation to each other and the BamHI sites, DNA digested with HindIII and EcoRI was also analyzed (Fig. 21 p 107). This yielded fragments of approx. 23.0, 1.4, 1.3, and 0.5 Md (Table 12). Since the 1.3 Md EcoRI fragment and the 1.4 Md HindIII fragment were cleaved out unchanged with the combination of these two enzymes, it can be concluded that cleavage sites for HindIII and EcoRI do not overlap. Furthermore, because a new 0.5 Md fragment (Fig. 21 p 107) appeared from this double digest, the 1.3 Md EcoRI fragment must be 0.5 Md away from the HindIII 1.4 Md fragment. As inferred before, HindIII has one cleavage site within the BamHI 4.0 Md fragment. This HindIII site must therefore be 1.0 Md away from one terminus of the BamHI 4.0 Md fragment. In this manner, the relative positions of the cleavage sites for HindIII, BamHI and EcoRI could be ordered. The type of analyses that were described here in some detail for three restriction endonucleases was extended to include five more endonucleases, SmaI, KpnI, SalI, BglII and PstI. From the

FIGURE 22. Restriction endonuclease cleavage map of pCU1 plasmid.

results of this restriction enzyme analysis the physical map of pCU1 shown in Fig. 22 could be constructed. From the addition of the molecular weights of the fragments generated with each of the various endonucleases, it was determined that pCU1 DNA had a molecular weight of 26.0 ± 0.3 Md (Table 12). This value is in excellent agreement with the molecular weight of pCU1 DNA as determined by electron microscopy (Fig. 2).

d) Localization of plasmid-associated phenotypes and confirmation of the cleavage map using deletions generated in vitro

In order to confirm the restriction endonuclease cleavage map and to localize some of the pCU1 specified phenotypes, deletion mutants of pCU1 were generated in vitro. Deletions spanning different regions of pCU1 DNA were generated by digesting the DNA separately with different restriction enzymes and using such DNA to transform the strains C600 or 490. Transformants were selected for either Ap^r , Sm^r or Sp^r and then screened for the absence of other plasmid-linked markers. Plasmid DNA was isolated and characterized from different selected classes of transformants. Table 4b describes the properties of the resulting plasmid deletion mutants. These plasmid deletion mutants were used to localize genetic regions of pCU1 on its physical map. The deletion mutant pCU16 has a molecular weight of 24.6 Md and upon cleavage with HindIII does not yield the 1.4 Md fragment which its parent pCU1 yielded (Fig. 23). Since bacterial

FIGURE 23. Single digests of plasmid DNA of pCU1 and two of its in vitro-generated deletion mutants, pCU10 and pCU11. A, B, C, D and E represent restriction fragments generated by digestion of pCU1, pCU10 and pCU11 DNA with single nucleases. In each case (from left to right) the pattern of fragments obtained for pCU1 DNA is followed by those obtained from pCU10 and pCU11 DNAs. Restriction endonucleases used are as follows: (A) BamHI, (b) BglIII, (C) SalI, (D) KpnI, (E) SmaI.

A

B

C

D

E

strains harbouring pCU16 were phenotypically indistinguishable from strains carrying pCU1 (Table 4b), it was inferred that removal of the HindIII 1.4 Md fragment did not alter any of the known pCU1-associated phenotypes. Mutant pCU10 has a molecular weight of 18.6 Md. It retains the markers Ap^r , Sm^r and Sp^r but does not confer conjugal transfer ability or the related function of Ike-sensitivity. This mutant has lost the KpnI generated 3.8 and 3.0 Md fragments. Simultaneously, one SalI and one SmaI site have been removed (Fig. 23). Some genes conferring the Tra^+ and Ike^s phenotype must therefore reside in the lost region of DNA. To obtain more information concerning these regions, the SalI-derived mutants pCU11 and pCU13 were used. DNA from these two plasmids and from pCU10 were analysed physically in the same manner that has been described for pCU1 (Fig. 24). The phenotypes conferred by these three plasmids are similar, i.e., they do not confer Tra^+ and Ike^s . Comparing the location and extent of deletions in the DNA of these three plasmid mutants (Fig. 22), it is apparent that a common region has been deleted, a region that includes the SalI-2 fragment (Fig. 22). Some genes conferring Tra^+ and Ike^s are therefore likely to be located in this region. It is not possible to conclude how far and in what direction from this common region, the Tra^+ and/or Ike^s region continues. It certainly does not extend into the HindIII-2 fragment as loss of this fragment in mutant pCU16 did not affect these two phenotypes.

FIGURE 24. 1.3% agarose gel electrophoresis of pCU1, pCU10 and pCU11 with a combination of two nucleases. For comparison, single digests for each of the two nucleases used is also included.

(A) Cleavage with KpnI + PstI. Slot 1, pCU1 digested with KpnI alone; slots 2, 3, 4, pCU1, pCU10 and pCU11 plasmid DNAs digested with KpnI + PstI; slot 5, pCU1 DNA cut with PstI alone.

(B) Cleavage with KpnI + HindIII. Slot 1, pCU1 DNA cut with HindIII alone, slot 2, 3 and 4, pCU1, pCU10 and pCU11 DNAs cut with HindIII + KpnI; slot 5, pCU1 DNA digested with PstI alone.

(C) Cleavage of plasmid DNA with a combination of KpnI + BamHI. Slot 1, pCU1 DNA cleaved with BamHI alone; slots 2, 3 and 4, pCU1, pCU10 and pCU11 DNAs cleaved by BamHI + KpnI.

(D) Cleavage of plasmid DNA with SalI + PstI. Slot 1, pCU1 DNA cut with SalI alone; slot 2, 3 and 4, pCU1, pCU10 and pCU11 plasmid DNA cut with SalI + PstI; slot 5, pCU1 DNA cut with PstI alone.

D

C

B

A

pCU12 is the smallest of the deletion derivatives (Figs. 22 and 25). This plasmid was obtained by treating pCU1 DNA with SmaI, an enzyme that produces blunt ends (Endow and Roberts, 1977) and using this preparation to transform *E. coli*. Since *E. coli* ligase does not have the ability to ligate blunt ends of DNA (Heffron *et al.*, 1978), this situation presumably favoured the *in vivo* extension of the deletion initially produced *in vitro* by SmaI. DNA included in this plasmid (7.0 Md) specifies all genes required for the stable maintenance of the plasmid and in addition the genes specifying resistance to the three antibiotics. By comparing the cleavage map of pCU12 to those of the other deletion mutants (Fig. 22), it is possible to narrow the region responsible for stable plasmid maintenance. The loss of HindIII-2 (in pCU16), EcoRI-2 (in pCU8) or SalI-2, 3 and 4 (in pCU11 and pCU13) does not interfere with plasmid maintenance. If these regions are excluded from pCU12, we are left with a 2.7 Md region which includes the junction of BamHI-1 and BamHI-2. This junction is present in all of the deletion mutants signifying the importance of this region for plasmid maintenance. Attempts to obtain deletion mutants of pCU1 after BamHI treatment were not successful. Such experiments did not yield any Ap^r transformants and the Sm^r or Sp^r transformants that arose were unstable. It is possible that cleavage of DNA at the BamHI-1-2 junction destroys a function that is important for some aspect of plasmid maintenance. The fact that in these experiments as well as in the experi-

FIGURE 25. Electron micrograph showing double-stranded molecule of pCU1 and pCU12. Electron microscopic determinations of molecular weights of pCU1 and pCU12 were 26.0 ± 0.5 Md and 7.0 ± 0.9 Md respectively.

ments using other restriction endoculeases on pCU1 DNA, deletions leading to Ap^S have not been obtained further suggests that the Ap^R gene must be in the vicinity of the region of the plasmid DNA conferring stable maintenance of the plasmid. A functional map of pCU1 which summarizes the results of these experiments is shown in Fig. 26.

V. Additional analysis of the Tc^R determinant in pCU7 and pCU4 plasmids

The results of the genetic and physical analysis of pCU4 and pCU7 plasmids were highly suggestive that the Tc^R determinant is part of a transposable element. Tc^R was a marker originally associated with pCU2 plasmid. By some kind of recombination reaction between pCU1 and pCU2, this marker became associated with pCU1 or pCU1-related plasmids. In order to confirm directly that the Tc^R region on pCU4 and pCU7 plasmids had its origin in pCU2 DNA-DNA hybridization experiments using the technique developed by Southern *et al.* (1975) was used. This procedure involved the separation of the enzyme-generated fragments by electrophoresis on agarose, the denaturation within the gel of the resulting resolved fragments, their transfer to a nitrocellulose filter, and hybridization with a radioactively labelled denatured, "probe" DNA to indicate regions of nucleotide sequence homology. The autoradiography of the filter allowed the visualization of DNA fragments which hybridized with the "probe" DNA. Details of this procedure are described in Materials and Methods and by Southern (1975).

FIGURE 26. Physical and genetic organization of pCU1 plasmid.

Both pCU4 and pCU7 were labelled in this manner and used separately as probes with restriction endonuclease digests of pCU2 DNA fixed to the filter. EcoRI digest of pCU4 or pCU7 DNA was present as well on each filter to serve as a positive control for hybridization. Fig. 27 represents the observed findings. In the case when pCU4 (Fig. 27 B-a) or pCU7 (Fig. 27 A-a) DNA was used as a "probe" a high degree of self-hybridization seen as a smear was detected. When the radioactive "probe" DNA was added to the filter charged with the pCU2 DNA cleaved by two or three restriction endonucleases, only certain pCU2 DNA fragments showed hybridization with pCU1-related plasmids. In the case of EcoRI-digest of pCU2, out of 16 bands only 2 carry sequences which find homology with either pCU4 or pCU7 DNA (Fig. 27 A-b' and B-a'). These bands correspond to two extra bands which are visible in the EcoRI-digest of pCU4 or pCU7 DNA but not in the same digest of pCU1 plasmid (Fig. 6A p 80).

Thus, the results obtained from the DNA-DNA filter hybridization showed that the genetic region bearing Tc-resistance in pCU4 or pCU7 plasmid finds homology with pCU2 DNA. This observation supported the notion that the Tc^r originally present in pCU2 has been acquired by pCU1 plasmid by some kind of genetic recombination. pCU1 and pCU2 although unrelated (Fig. 10 p 88) both carry the Sm^r gene which might potentially serve as a region of homology required for general recombination. The results of the experiments presented in this section eliminate the possibility that the

FIGURE 27. Autoradiograph of DNA hybridization with ^{32}P -labelled pCU7 (A) or pCU4 (B).

A - agarose gel electrophoresis of:

- a) pCU7 cut with EcoRI
- b) pCU2 cut with EcoRI
- c) pCU2 cut with BamHI

a', b' and c' are autoradiographs of nitrocellulose filter after hybridization with ^{32}P -labelled pCU7 DNA.

B - agarose gel electrophoresis of:

- a) pCU4 cut with EcoRI
- b) pCU2 cut with EcoRI
- c) pCU2 cut with BamHI
- d) pCU2 cut with XhoI

a', b', c' and d' represent autoradiographs of nitrocellulose filter after hybridization with ^{32}P -labelled pCU4 DNA.

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hybridized region represents Sm^r , since pCU7 is a Sp^s and Sm^s deletion mutant. It is inferred that pCU7 originated by a deletion of Sp^r and Sm^r from pCU4 in the same manner as pCU8 was derived from pCU1 plasmid. It is possible that Sm^r in pCU1 or pCU2 might originally have served as a region of homology. In order to rule out this possibility it was important to determine whether Tc^r was inserted in the vicinity of Sm^r in pCU1.

The position of the region carrying Sm^r in pCU1 DNA has been determined earlier (Fig. 26). It lies within the EcoRI-2 fragment. The observation from the electron microscopic analysis of pCU1 and pCU8 plasmid molecules revealed that the loss of the 1.7 Md stem-loop structure (C) can be linked with the phenotypic loss of Sp^r and Sm^r . Thus, this structure should be within the EcoRI-2 fragment. Furthermore, the EM analysis indicated that the stem-loop structure A into which the Tc^r inserted is 1.5 Md away from the stem of structure D. It is possible to calculate the distance between structure C and D (Fig. 12 p 92); structure C and D are approx. 8 Md away from each other. This intervening region has a point into which Tc^r has inserted. If the structure C is fixed within the EcoRI-2 fragment then the 8 Md region might go either to the left of it (into Sall-2 fragment on pCU1 map) or to the right of it (into EcoRI-1 fragment). In order to exclude one or the other of these two possibilities, pCU4 DNA was digested with Sall and transformed. This gave rise to a deletion mutant of pCU4 which was named pCU13. It

carries resistance to Ap, Sm, Sp but was Tc^S . It was also Tra^- and IKe^R . The plasmid DNA of pCU13 was purified and digested with BamHI, EcoRI, HindIII and SalI. Fig. 28 displays the pattern obtained from pCU13 and pCU4 after the electrophoresis of DNA fragments. A comparison of the restriction patterns of pCU4 and pCU13 show that the fragments representing Tc^R in pCU4 (see Fig. 4 p 75) are no longer present. This is the case for all of the enzymes used (e.g. BamHI fragments 2 and 4; EcoRI fragments 2 and 3; HindIII fragment 2 and also 3; SalI fragments 2-3 and 4).

This comparative analysis thus indicates that the deletion region does not affect the EcoRI-2 fragment on which Sm^R is localized. It appears that the Tc^R is inserted in either SalI fragments 2-3 and 4. To determine unambiguously the point of insertion, pCU4 DNA was cleaved with two enzymes simultaneously. EcoRI and HindIII nucleases were used in a double digest reaction (Fig. 29). HindIII digest of pCU4 (Fig. 4 p 75) generates just one new fragment as compared to the digest of pCU1 using the same enzyme (Fig. 4 p 75). Therefore, HindIII cleaves just at one point of the inserted region. The size of this new fragment is 9 Md. It thus appears that the new cleavage site is 9 Md away from the HindIII-2 fragment of pCU2. EcoRI enzyme shows two additional fragments in pCU4 as compared to the same digest of pCU1 (Fig. 4 p 75). In accordance with this there are two sites for EcoRI nuclease within the inserted region. The double digest of HindIII-EcoRI shows that the EcoRI-2 fragment

FIGURE 28. 1% agarose gel electrophoresis of DNA fragments generated from either pCU4 (left digest in each panel) or pCU13 DNA. From left to right.

Panel A: pCU4 and pCU13 digested with BamHI

Panel B: pCU4 and pCU13 digested with EcoRI

Panel C: pCU4 and pCU13 digested with HindIII

Panel D: pCU4 and pCU13 digested with SalI

D

C

B

A

FIGURE 29. 0.7% agarose gel electrophoresis of the pattern generated by EcoRI, EcoRI + HindIII and HindIII from pCU4 (A) and pCU1 (B) DNA.

A - cleavage of pCU4 DNA by:

- a - EcoRI alone
- b - EcoRI + HindIII double digest
- c - HindIII alone

B - cleavage of pCU1 DNA by:

- a' - EcoRI alone
- b' - EcoRI + HindIII double digest
- c' - HindIII alone

The digests of pCU1 have been included for comparison.

A

a b c

B

a' b' c'

carries a cleavage site for HindIII. This fragment is shorter, and has a molecular weight of 4.7 Md. This EcoRI-2 which comes from the Tc^r region is 4.0 Md away from HindIII-2 fragment ending into Sall-2 fragment on the pCU1 map (Fig. 30). Since the molecular weight of the inserted DNA sequence has been estimated to be 8.0 Md, the position of the 4.7 Md EcoRI fragment coming from the inserted region is within the cross-hatched area in Fig. 30. Other restriction endonucleases cutting within the inserted region are: BamHI-2x; Sall-2x; SmaI-2x; PvuI-4x; PstI-, KpnI, BstEII, BglII, HpaI (where x, represents a cutting site).

It is concluded that the Tc^r determinant initially present in pCU2 plasmid became associated with pCU1 and that such an association arose by a recombination reaction that did not occur in the Sm^r region of the two plasmids, a region that could conceivably have provided sufficient homology for general recA-promoted recombination. The association arose probably by the translocation of a segment of DNA carrying Tc^r from pCU2. The evidence presented is highly suggestive that the Tc^r marker is part of a transposable element. However, the attempts to translocate the Tc^r marker from pCU4 to phage λ b221c1857 (Kuntz, B.Sc. Hons. Thesis, Carleton University, 1980) have been unsuccessful. Possibly explanations for these negative results will be discussed.

FIGURE 30. Diagram showing the location of the insertion of the Tc^r region from pCU2 into pCU1 to create pCU4. Shaded area represents the possible location of the rest of the "tetracycline resistant transposon" (i.e., 8 Md less the 5.9 Md EcoRI fragment).

DISCUSSION

Sensitivity to bacteriophage IKE is widely considered to be a property that is diagnostic for the presence in strains of Enterobacteriaceae of plasmids belonging to the Incompatibility Group N. The plasmid RM98 was isolated and described as such a plasmid (Khatoon and Iyer, 1971). However, during the course of this investigation it has been shown that the composition of different RM98⁺ strains may not be the same, although their overt plasmid-phenotypes were similar. The genealogically older RM98⁺ strain carried two different conjugative plasmids. One of the two plasmids belongs to the N group and specifies resistance to Ap, Sm, Sp, transfer proficiency and sensitivity to bacteriophage IKE and PRD1. This plasmid has been designated pCU1. The second plasmid designated pCU2, specifies resistance to Sm, and Tc. It is also a conjugative plasmid but not of the N group. The genetic evidence indicates that pCU2 specifies a different incompatibility system and also a different conjugative system from that specified by the N group plasmid pCU1. pCU1 and pCU2 are both transmissible plasmids. Among R plasmids, conjugative ability is generally specified by a group of genes comprising a significant portion of the plasmid genome (Barth et al., 1978; Barth, 1979; Silver et al., 1977; Tanaka et al., 1976). pCU1 and pCU2 plasmids have been shown to share very little homology in the DNA-DNA hybridization experiments. On the basis of this evidence and also the results of the sensitivity to different sex-specific bacteriophages it is concluded that pCU1 and pCU2 specify different conjugative systems. While

pCU1 belongs to the conjugative group N, pCU2 probably specifies a novel conjugative system among Enterobacteriaceae. A study of this plasmid would be of interest but it has not formed the subject of this thesis. Information obtained during the analysis of the DNA of the two plasmids also indicated that they were dissimilar. The contour length of molecules of pCU2 was much longer than that of pCU1 molecules. The electrophoretic pattern obtained after the cleavage of pCU1 or pCU2 DNA with several restriction-endonucleases, further confirmed that pCU1 and pCU2 are different plasmids. In spite of this dissimilarity, recombination reactions appear to occur between these two plasmids. Such a recombination event has generated a new N group plasmid, pCU4 which carries all of the markers initially present in pCU1 and in addition the Tc^r marker of pCU2. The filter hybridization experiments showed directly that sequences comprising the Tc^r marker in pCU4 have complementary regions in pCU2 and therefore probably arise from the latter. In conjugation experiments, involving the original RM98⁺ strain carrying the plasmid aggregate of pCU1 and pCU2 there is a selection for the product of such recombinant plasmids because pCU1 has the more efficient conjugative system. Selection for Tc^r following conjugation therefore favours the isolation of such derived plasmids. From the observation that pCU1 and pCU2 are dissimilar and that pCU1 acquires a discrete piece of DNA, concurrently with the acquisition of Tc^r , it is suggested that the Tc^r gene is part of a transposable element. This

is further supported by the detection of an additional stem-loop structure in Tc^r derivatives of pCU1. This large stem-loop structure has been shown to be inserted within a region containing a different stem-loop structure in the pCU1 molecule. In some other cases described in the literature evidence has been presented that the termini of one transposable element can serve as "hot spots" for the integration of other transposable elements (Kopecko et al., 1976; Laufs et al., 1979). Attempts to translocate Tc^r from pCU4 into bacteriophage λ b221c1857 were unsuccessful (Kuntz, B.Sc. Hons. Thesis, Carleton University, 1980). One possible explanation might be the observed integration of Tc^r within the other inverted repeat loop structure present on the pCU1 molecule. This results in the formation of a very large stem-loop structure, which totals a length that far exceeds the capacity of λ b221c1857 to incorporate it and package it into its capsid (Berg, 1977). Tetracycline-resistance determinants on some R plasmids have been shown to be part of transposable elements. Three different kinds of Tc^r translocons have been described (Kleckner et al., 1975; Schmitt et al., 1979; Schöffl and Pühler, 1979a, b). The Tn10 transposon has a molecular weight of 6.2 Md and thus is smaller than the Tc^r transposon present in pCU2 and pCU1-related plasmids. The other two Tc^r transposons Tn1721 and Tn1771 described in the literature are very similar to each other, their size (7.2 Md) is slightly smaller than the size of the stem-loop structure associated with Tc^r and observed in pCU4 or pCU7.

molecules. The comparison of restriction endonuclease cleavage sites in Tn10, Tn1721 (Tn1771) and the Tc^r transposon from this study, further suggest that they are different. While Tn10 contains cleavage sites for BamHI, AvaI, BglI, BglIII, EcoRI, XbaI, HincII, HindIII and HpaI, the restriction enzymes PstI, SmaI, KpnI, XhoI, SalI and PvuI do not cleave within the Tn10 element (Jorgensen et al., 1979). The Tc^r region on pCU4 plasmid is cleaved by all the enzymes of the latter group except XhoI. The Tc^r transposon described here also differs from Tn1721 (Tn1771). It has only two EcoRI cleavage sites while Tn1721 (Tn1771) has three. Furthermore, it does not show a selective amplification of the Tc^r region as the two other transposons do (Schmitt et al., 1979; Schöffl and Pühler, 1979a, b). It thus appears that the tetracycline-resistance on pCU2 and pCU1 related plasmids represents a new transposon. In creating plasmids such as pCU4 and pCU7, this element contributes to genetic and physical rearrangement of plasmid DNA.

In the strain carrying the plasmid aggregate, the N group plasmid pCU1 is itself spontaneously unstable. Changes in the molecular structure of pCU1 have been shown to result from deletions (pCU8), addition (pCU4) or a deletion and an addition (pCU7) of antibiotic resistance genes. In principle, this instability of pCU1 could have been a consequence of interactions with the second plasmid pCU2 (e.g. interactions involving the Tc^r transposon). However, it has been demonstrated that in some derived strains with altered forms of

pCU1, pCU2 is absent (e.g. pCU8, pCU7) and in others it is present (e.g. pCU9). This has suggested that the physical and genetic changes in pCU1 are not necessarily coupled to interactions with pCU2 and that some instability may be an inherent feature of pCU1. The observed step-loop structures on pCU1 molecules might contribute to structural instability. The spontaneous loss of Sm^r and Sp^r in pCU8 was shown to be a consequence of a loss of one of these stem-loop structures. Other stem-loop structures have also been observed on single-stranded molecules of pCU1. At the present time, functions have not been assigned to these structures. All of these structures are close to each other. One of them, structure B, has been observed, to be inserted within the larger structure A. Such naturally occurring "double-loop" structures may only be compared to Tn4 (Kopecko et al., 1976). In the case of Tn4 however, evidence has been presented indicating that one of the transposable elements specifying Ap^r is contained within the length of another transposon specifying Sm^r and Su^r . This situation is somewhat similar to the insertion of Tc^r into the loop of structure A. One of the interesting features of the stem-loop B is its small size. The smallest translocatable element described is Tn204 (Kleckner, 1977) which is much bigger than the structure B. It would therefore be interesting to determine what kind of genetic functions are encoded within structure B.

A further development from this study was the construction of a restriction endonuclease-cleavage map of the IncN

group plasmid pCU1 and its various deletion mutants (Fig. 31). As determined by the summation of the sizes of the fragments generated from this DNA upon complete digestion with eight different restriction endonucleases, the molecular weight of the plasmid is 26.0 ± 0.3 Md which is in agreement with the molecular weight of the plasmid as determined by the measurement of the contour length of intact DNA molecules. For EcoRI, there are two cleavage sites on pCU1 both of which are in the region specifying Sm^r and Sp^r . For six other plasmids of the IncN group (RN3, N3T, RCP3, pIP113, TP118, pKM101), EcoRI has been found to cleave the DNA at only one or two sites (Chabbert et al., 1979; Langer et al., 1981). A similar paucity of EcoRI cleavage sites has been observed for those IncP and IncW groups of plasmids that have been studied (Chabbert et al., 1979). It also appears that those few EcoRI-cleavage sites that are found on these plasmids tend to be associated with regions specifying antibiotic resistance (Langer et al., 1981, and this study). For those plasmids of these three groups (Barth et al., 1978; Langer et al., 1981) where information on the genetic organization of the plasmid is available (RP4, pKM101, pCU1), EcoRI sites are absent from their conjugal transfer regions. This is clearly not the case for plasmids such as F, R1dtd-19 and R100 that determine the better-known F transfer system (Skurray et al., 1978; Willetts and Skurray, 1980; Blohm and Goebel, 1978; and Tanaka et al., 1976). These observations suggest that there has been some conservation of nucleotide

FIGURE 31. Restriction endonuclease cleavage maps of pCU1 and its various deletion mutants.

sequences in the transfer regions of IncN, P and W groups of plasmids, a suggestion which is consistent with physiological observations on bacteria carrying any one of these plasmids. Thus, there are bacteriophages described with a host range that is broad enough to include all three of these groups but to exclude other groups (Olsen and Shipley, 1975; Bradley and Rutherford (1975) and bacteria carrying a conjugative plasmid of any one of these groups have been found to be able to kill a strain of Klebsiella pneumoniae (Rodriguez and Iyer, 1981). In the IncP group plasmid RP4, there is evidence indicating that the tra genes are interspersed between other genes in three separate groups (Barth and Grinter, 1977; Barth et al., 1978; Barth, 1979). This does not appear to be the case for the IncW group plasmid R388 (Ward and Grinstead, 1978) or the IncN group plasmids pKM101 and pCU1 (Langer et al., 1981, and this study). No deletion mutant of pCU1 that had lost its ability to confer transfer function was able to confer sensitivity to either IKE or PRD1. Thus sensitivity to these two phages appears to be included within the Tra region. While this study locates the Tra region on pCU1, it does not define precisely the limits of this region.

As in the case of many other antibiotic-resistance plasmids, in pCU1, genes for antibiotic-resistance are clustered at a position away from the transfer region. However, while genes specifying Sm^r and Sp^r could readily be deleted to give rise to viable plasmid mutants, the deletion of Ap^r could not be observed. The restriction endonuclease-

3 3
OF / DE

cleavage and functional maps of pCU1 indicate that the basis of this stability of Ap^r is its location in the vicinity of regions conferring plasmid replication and segregation, a region that we refer to as the plasmid maintenance region.

A minimum estimate of this region including Ap^r is 2.7 Md.

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