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THE N SYSTEM OF BACTERIAL CONJUGATION: GENETIC ANALYSIS AND
RELATIONSHIP TO PHAGE SENSITIVITY AND THE KILLING
OF KLEBSIELLA PNEUMONIAE

by

Vinay Thattai, 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

Department of Biology
Carleton University,
Ottawa, Ontario.

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ABSTRACT

Conjugative antibiotic resistance plasmids of the incompatibility group N (IncN) have a wide host range and confer upon their bacterial hosts sensitivity to the bacteriophages IKE and PRD1 and the ability to kill Klebsiella pneumoniae when conjugated with the latter. In this study, the regions on a particular IncN group plasmid pCU1 that determine these plasmid-associated phenotypes have been analysed using a variety of molecular genetic techniques and concepts - restriction endonuclease mapping, deletion mapping, transposon mutagenesis, molecular cloning and genetic complementation.

A continuous 19.4 Kb region when cloned into the small non-transmissible plasmid pACYC184 conferred self-transmissibility and phage sensitivity to the strain carrying it. This tra region was cloned by introducing HindIII sites artificially into regions of the plasmid where such sites do not normally exist. A large number of Tn5 insertions were isolated and mapped to regions of the plasmid, both at the ends of this 19.4 Kb tra region as well as within it. The phenotype affected by these transposon insertions and of subclones and deletions derived from plasmids carrying them, reduced the tra region to 16 Kb. A subset of this region (11.2 Kb) at one end of tra determined IKE and PRD1 sensitivity. Insertions at the other end abolished the Tra phenotype

but not phage sensitivity. This 5.4 Kb region was inferred to be concerned with some other aspect of conjugation. The region required in cis for plasmid transfer (mob region) was localized to a 1.2 Kb sequence at the end of tra away from the pilus region.

A large number of Tn5 derivatives of pCU1 and of a tra-containing clone were used for a genetic complementation analysis of the tra region. Using two different genetic complementation tests, it was concluded that there are at least eight complementation units within tra, six of which were localized to the pilus region. This is the minimum number of tra complementation units since there were two regions (1.3 Kb and 2 Kb in size) within the 16 Kb tra region where none of the 117 insertions that were used, mapped.

The plasmid carrying the cloned 19.4 Kb tra region did not mediate killing of K. pneumoniae. For this killing to occur an additional 3 Kb region beyond tra at the mob end needed to be cloned. On the basis of this observation and the isolation of Tn5 insertions at the opposite end of tra which led to a Kil^- but Tra^+ phenotype, it was concluded that two separate regions flanking tra were both needed for this phenotype.

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TO MY FATHER

TABLE OF CONTENTS

Abstract.....	i
Acknowledgements.....	iii
Dedication.....	iv
TABLE OF CONTENTS.....	v
LIST OF FIGURES.....	ix
LIST OF TABLES.....	xi
LIST OF ABBREVIATIONS.....	xii
INTRODUCTION.....	1
REVIEW OF THE LITERATURE.....	7
The F transfer system.....	9
I) Genes and gene products of the system.....	9
a) Properties of F.....	9
b) Genetics of conjugation.....	10
c) Order of <u>tra</u> genes and direction of transcription.....	14
d) Molecular cloning of regions of F- <u>tra</u>	15
<u>in vitro</u> cloning.....	15
<u>in vivo</u> cloning.....	20
e) Correlation of genetic and physical map...21	
f) <u>tra</u> gene products.....	22
g) F-pilus biochemistry.....	24
h) Regulation of transfer.....	25
II) The process of conjugation.....	26
a) Cell-to-cell interaction.....	26
b) Sex-pili (Pili).....	28
c) Role of pilus in transfer.....	30

d) Formation and stabilization of mating aggregates.....	33
e) Conjugal DNA metabolism.....	34
f) Origin of transfer and nicking of DNA at the origin.....	36
g) Mobilization of non-conjugative plasmids and specificity of <u>oriT</u>	38
h) Transfer of DNA.....	41
i) DNA synthesis for conjugal DNA transfer...	44
j) Barriers to conjugation.....	46
The P transfer system.....	49
The W transfer system.....	54
Other transfer systems.....	56
MATERIALS AND METHODS.....	59
Bacterial strains, plasmids and bacteriophages.....	60
Media and Chemicals.....	60
Construction of strains carrying plasmids.....	63
Test for <u>recA</u> mutation.....	64
Determination of the frequency of transfer.....	64
Determination of the frequency of Killing of <u>Klebsiella pneumoniae</u>	65
Determination of plasmid associated phenotypes...	65
Procedures involving the use of bacteriophages...	67
Phage lysate of IKE and PRD1.....	67
Preparation of λ ::Tn5 lysate.....	68
Plasmid DNA manipulations.....	68
Isolation of plasmid DNA.....	68

Boiling method.....	68
Rapid alkaline extraction procedure.....	69
Clear lysate procedure.....	70
Genetic transformation.....	73
Restriction endonuclease digestion and electrophoresis of DNA fragments.....	74
Isolation of deletion mutants of a plasmid.....	76
Cloning of plasmid DNA fragments.....	77
Selection of Tra ⁺ recombinant clones.....	77
Isolation of transposon Tn5 derivatives.....	78
Genetic complementation analysis.....	79
Transient heterozygosis technique.....	80
RESULTS.....	82
A) Approximate location of N- <u>tra</u> on the pCU1 physical map.....	84
B) Cloning of BglIII fragments of pCU1.....	89
C) Isolation of transposon insertions.....	97
D) Mapping Tn5 insertions on the pCU1 map.....	102
E) Localization of the ends of the <u>tra</u> region.....	107
F) Cloning of a minimal region that includes <u>tra</u>	110
G) Localization of <u>tra</u> within the estimated 16 Kb <u>tra</u> region (between 31.5 Kb and 8.2 Kb).....	115
H) Attempts to saturate the 9 Kb central region of <u>tra</u> with transposon Tn5	116
I) Localization of the region determining N-pilus-specific phage sensitivity.....	119
J) Localization of region required <u>in cis</u> for transfer (<u>mob</u>).....	123

K) Relationship of the <u>tra</u> region to the Kil phenotype.....	126
L) Genetic complementation analysis.....	137
M) Transient heterozygosis technique.....	143
DISCUSSION.....	146
APPENDIX I.....	159
REFERENCES.....	163

LIST OF FIGURES

Fig. 1	Genetic map of plasmid F.....	16
Fig. 2	Regulatory circuit of F transfer.....	27
Fig. 3	Map of RP4/RK2/RP1 showing the location of Tra regions.....	53
Fig. 4	Map of the IncW group plasmid S-a.....	57
Fig. 5	A map of plasmid pCU1 showing some of the restriction sites relevant to this study.....	86
Fig. 6	Restriction maps of some of the plasmids constructed in this study.....	88
Fig. 7	Scheme used for the molecular cloning of the BglII fragments of pCU1.....	90
Fig. 8	To determine the orientation of BglI-1 fragment of pCU1 with respect to pACYC184 in pCU53.....	95
Fig. 9	Partial restriction map of Tn5.....	99
Fig. 10	The different classes of pCU1::Tn5 derivatives obtained after transposon mutagenesis.....	101
Fig. 11	Restriction patterns generated by various enzymes for pCU1 and pCU29.....	104
Fig. 12	Tra ⁺ insertion derivatives of pCU1.....	109
Fig. 13	Scheme used to clone the region from pCU1 required for conjugal transfer into HindIII site of pACYC184.....	112
Fig. 14	Agarose gel electrophoresis of HpaI-digested DNA of pCU101, pCU105 and pCU109.....	113
Fig. 15	Map position of the Tra ⁺ pCU109::Tn5 derivatives on the physical map of pCU109.....	118
Fig. 16	Localization of the <u>mob</u> region of pCU1.....	122
Fig. 17	Position of Tn5 insertion on the pCU1 map of pCU57.....	127
Fig. 18	Electrophoretic migration patterns of restriction fragments generated from pCU167,	

pCU184, pCU66 and pCU1 with different enzymes.....	128
Fig. 19 Construction of plasmid derivatives to define the position on pCU1 of the two regions required for the killing of <u>K. pneumoniae</u>	130
Fig. 20 <u>kilA</u> and <u>kilB</u> regions required for killing of <u>K. pneumoniae</u>	135
Fig. 21 The position of the transposon Tn5 in the Tra- pCU1::Tn5 and pCU109::Tn5 derivatives...	142
Fig. 22 A comparison of the distribution of restriction sites in the transfer regions of the N group plasmid pCU1 and R46.....	150
Fig. 23 A combined physical and genetic map of pCU1...	157

LIST OF TABLES

Table 1	Bacterial strains.....	61
Table 2	Plasmids derived and used.....	62
Table 3	Recombinant clones derived from pCU1.....	92
Table 4	Fragment sizes generated by the digestion of pCU1 and pCU29 with different restriction endonucleases.....	106
Table 5	Comparison of the conjugal transfer frequencies of pCU1, pCU29 and pCU101.....	108
Table 6	The frequency of mobilization of various pCU1 derivatives by pCU1.....	125
Table 7	Comparison of the efficiency of plasmid transfer and of killing of <i>K. pneumoniae</i> by <i>E. coli</i> strains carrying pCU167, pCU184 pCU66 and pCU1.....	131
Table 8	Comparison of the conjugal transfer frequencies and the efficiency of killing of <i>K. pneumoniae</i> by strains carrying pCU1, pCU88 or pCU109.....	133
Table 9	Determination of Tra and Kil phenotypes of strains carrying pCU1, pCU101 and pCU105....	134
Table 10	Determination of functions required for killing of <i>Klebsiella pneumoniae</i>	138
Table 11	Genetic complementation analysis of the <u>tra</u> region using pCU1::Tn5 and pCU109::Tn5 derivatives.....	139-140
Table 12	Genetic complementation by the transformation mediated transient heterozygosis technique..	144

LIST OF ABBREVIATIONS

The genetic abbreviations are in accordance with the proposals of Novick et al. (1976) and Demerec et al. (1966). The symbols specifying the genotype are underlined.

-	Absence of phenotype
ATP	Adenosine triphosphate
<u>am</u>	Amber mutation
Ap	Ampicillin
bp	Base pairs
C	Celsius
cfu	Colony forming unit
Cm	Chloramphenicol
CCC	Covalently closed circular
DNA	Deoxyribo nucleic acid
DCDS	Donor Conjugal DNA Synthesis
ds	Double stranded
E	Electrophoresis buffer
Et-Br	Ethidium bromide
<u>E. coli</u>	<u>Escherichia coli</u>
EDTA	Ethylenediamine tetracetate
F	Sex factor
g	Acceleration due to gravity
gm	Grams
h	Hours
Hfr	High frequency of recombination
Inc	Incompatibility
Kb	Kilobase pairs
Kil	Killing of <u>Klebsiella pneumoniae</u>

Km	Kanamycin
<u>K. pneumoniae</u>	<u>Klebsiella pneumoniae</u>
λ	Bacteriophage Lambda
LB	Luria broth
mat	Maintenance
ug	Micro gram
ul	Microlitre
ml	Millilitre
moi	Multiplicity of infection
min	Minute
Nal	Nalidixic acid
nm	Nanometer
<u>ori</u> T	Origin of conjugative transfer
pfu	Plaque forming units
Pil	Pilus
PEG	Polyethylene glycol
+	presence of phenotype
r	Resistance
RCDS	Recipient conjugal DNA synthesis
Rif	Rifampicin
RNA	Ribonucleic Acid
sec	Seconds
s	Sensitivity
ss	Single stranded
SDS	Sodium dodecyl sulfate
Sm	Streptomycin
Sp	Spectinomycin
Tc	Tetracycline
TE	Tris-HCl, EDTA buffer

Tn	Transposon
Tra	Conjugal transfer
UV	UltraViolet light
u	Units
v	Volts
v/v	Volume per unit volume
w/v	Weight per unit volume

INTRODUCTION

Among the commonly recognized modes of gene transfer in bacteria (conjugation, transformation and transduction), bacterial conjugation is the most efficient mode (Low and Potter, 1978). The ability to transmit genes by mating or conjugation between bacterial cells is widely distributed (review by Rowbury, 1977; Willetts, 1981). Bacterial conjugation is a highly specialized process which requires cell-to-cell contact (Willetts and Skurray, 1980). In most such cases, the genes for conjugation are carried in the DNA of extrachromosomal genetic elements termed plasmids which are self-transmissible (Achtman and Skurray, 1977; Willetts and Skurray, 1980). Bacterial plasmids can be grouped into two major categories: conjugative and non-conjugative (Broda, 1979). A conjugative plasmid can bring about its own transfer and under certain conditions (when integrated into the chromosome to give rise to an Hfr strain) can transfer the host chromosome from one cell (donor or a male) to another (recipient or a female) (Lederberg and Tatum, 1946). A non-conjugative plasmid cannot bring about its own transfer. Even those plasmids that are not self-transmissible are frequently found to carry, in their DNA, regions such as the origin for conjugal transfer and mobilization that enable them to be transmitted into other cells with the help of self-transmissible plasmids present in the same cell (Dougan and Sherratt, 1977; Inselburg,

1977). A large number of naturally occurring drug resistance plasmids that have been isolated and described in the literature carry this property of self-transmissibility. Bacterial mating mediated by plasmids must therefore be a powerful force in bacterial evolution. To accomplish this, bacteria have evolved a number of different conjugative systems (Bradley et al., 1980). Naturally occurring plasmids have been also classified conveniently into several incompatibility (Inc) groups and those belonging to one Inc group generally possess a correlated conjugation system (Datta, 1975). Incompatibility is an operational term and defined as the inability of the two plasmids to be maintained in the same cell in the continued absence of selection pressure (Datta, 1975).

The existence of conjugative plasmids was first recognized in Escherichia coli K-12 by Lederberg and Tatum (1946) who discovered the plasmid F. Since then, a large number of conjugative plasmids have been found in a wide variety of bacterial genera (see Appendix B of Bukhari et al., 1977). At present, among the Enterobacteriaceae there are at least five conjugative groups that have been recognized. These are F, I, N, P and W (Willetts, 1977a). This classification is crude and is based only on the isolation of bacteriophages which are specific for any one group. Since phages that are specific for other Inc group

plasmids have been found and in some cases specific pili (see below) have been found on such plasmid-carrying cells (Bradley, 1980b; Bradley et al., 1982; Coetzee et al., 1982; 1983) it is probable that the number of tra systems now recognized is an underestimate.

All conjugative systems share three common features: a surface organelle called a pilus which is believed to be essential for initial contact between potential donor and recipient cells and to which male specific phages adsorb, a highly specialized system that encodes products required for the processing of DNA during plasmid transfer (DNA transfer metabolism) and surface exclusion, whereby the entry of the plasmid in the recipient is prevented by the presence of an identical or closely related plasmid (Datta and Hedges, 1971; Willetts, 1977a). In spite of these similarities, the conjugation systems specified by these groups are possibly quite dissimilar (Willetts, 1977a). It is known, for example, that the DNA of plasmids belonging to different conjugative systems do not share extensive regions of DNA homology (Sharp et al., 1973; Falkow et al., 1974) and that transfer deficient mutants of one incompatibility group (for example, Inc FI) are not complemented by the wild type plasmids of another Inc group, Inc I_a (Willetts, 1970; Cooke et al., 1970).

These observations suggest that even among the Enterobacteriaceae, a number of different conjugative transfer systems exist. At the level of genes or gene products, these systems may be totally independent of one another, genes or gene products of one system being incapable of substituting or interacting with those of another system. Alternatively, the transfer systems may show different types of interrelationship. To explore these possibilities, an analysis of at least one representative plasmid specifying each of the known tra systems needs to be undertaken. This study concerns the N-tra system about which little is known at the present time. The choice of this system was made because of the following observations.

- (a) Plasmids of the N-tra group are transmitted to a wider range of bacteria than other plasmids such as the intensively studied plasmid F and F-like plasmids possessing the F-tra system (Datta and Hedges, 1972; Tardif and Grant, 1980). There is some evidence to suggest that N-plasmids are related to two other Inc group plasmids P and W, since bacteriophages have been isolated whose host-range includes all the three groups (Olsen et al., 1974; Coetzee et al., 1979). While the nature of this relationship is not understood, taken together the host-range of these plasmids extends beyond the Enterobacteriaceae (see Bukhari et al., 1977).

(b) LKe, a bacteriophage, can plaque on cells carrying conjugative N-group plasmids only (Khatoon et al., 1972); the receptors for this phage are on pili which are short and pointed (Bradley, 1979) and which are serologically distinct from pili elaborated by representatives of other Inc groups of plasmids (Bradley, 1980a).

(c) Plasmids of this group have the ability to mediate killing of certain strains of Klebsiella pneumoniae, this require efficient plasmid transfer (Rodriguez and Iyer, 1981a; 1981b).

At present most of our understanding of conjugation comes from detailed studies over the last three decades on the F tra system. Comparatively, our knowledge of other tra systems is primitive although there have been some recent studies on aspect of the tra of IncH1, IncI, IncJ, IncN, IncP and IncW group plasmids (Taylor, 1983; Boulnois and Wilkins, 1979; Nugent, 1981; Winans and Walker, 1983; Lanka and Barth, 1984). While there must be differences between different tra systems, it is likely that what we learn from the development of knowledge on F tra system will be instructive in researching and understanding these other systems. I have therefore chosen to review historically, the F tra system in detail while not ignoring available information on the other tra systems.

REVIEW OF THE LITERATURE

Bacterial conjugation was initially demonstrated by the appearance of prototrophic strains of Escherichia coli K-12 when two auxotrophic mutants were mixed together (Lederberg and Tatum, 1946). This property was first attributed and then demonstrated to arise because of the presence of a bacterial sex factor called F (for fertility) in one strain (Hayes, 1953). However, it has since been found that this property is not unique to F or to E. coli. It has been demonstrated in a wide variety of bacteria and shown to be conferred by a large number of different sex factors or conjugative plasmids of which F happened to be an example (Rowbury, 1977). For practical purposes and because transfer can be a low frequency phenomenon, it is difficult to demonstrate the transfer of a plasmid unless it is genetically linked to some easily selectable phenotype. A large number of phenotypes associated with plasmids are now recognized; resistance to antibiotics, fermentation of carbohydrates and determinants of pathogenicity can be cited as examples. Conjugative plasmids linked to antibiotic-resistance markers are called R-factors (Mitsuhashi, 1971). To distinguish between F and R-factors, they were originally classified by their ability or inability to inhibit the fertility of F i.e. F_i^+ (Fertility inhibition) and F_i^- respectively (Meynell et al., 1968). Since this classification was found to be inadequate, plasmids were

classified on the basis of incompatibility. At present there are at least 30 Inc groups that are recognized in E. coli K-12 (Datta, 1979). The F type of conjugation (=F tra system) is not only coded by F itself but also by some other naturally occurring conjugative plasmids that fall into at least four different Inc groups.

The F tra system

I) Genes and gene products of the system

a) Properties of F.

F is isolated from cells as a covalently closed circular (CCC) DNA molecule. Its size was originally calculated as 94.4 kilobase pairs (Kb) (Sharp et al., 1973; Davidson et al., 1975). This was adjusted to 100 Kb based on more refined mapping techniques (Johnson and Willetts, 1983). It exists in 1-2 copies per E. coli K-12 host genome. Occasionally, it is covalently linked to the bacterial chromosome by general recombination owing to homology provided by the presence of at least three distinct Insertion Sequences (IS), IS3, $\gamma\delta$ (Tn1000), IS2 present both on F and the chromosome (Davidson, 1975; Hu et al., 1975). In this integrated state called an Hfr, (for high frequency of recombination) it allows the polarized transfer of chromosomal genes from one bacterium to another. F can excise precisely or imprecisely (Davidson et al., 1975). When excised imprecisely it can carry neighbouring chromosomal genes. Such F factors are

designated as F' (F-prime). The genetic analysis of transfer was carried out using F and F' factors.

b) Genetics of conjugation.

Genetic analysis of the F tra system was started with the isolation of mutants of the F' factor F'8-gal (Ohtsubo, 1970). These mutants were isolated by mutagenesis of cells carrying F' plasmids, by mutagenesis of an Hfr strain with nitrosoguanidine or by selecting for cells resistant to the F-specific phage M12. The mutants obtained were classified into different classes on the basis of sensitivity to other male specific bacteriophages. A large number of them were resistant to the F specific phages, M12, MS2, f2, QB, F1 and ZD (class I). Some mutants had retained sensitivity to QB, F1 and ZD but had lost sensitivity to others (class II). Class I did not produce F pili which are specific proteinaceous structures extending out from the bacterial cell. However, mutants showing sensitivity to all phages (class III) and of class II did produce F-pili to which all RNA phages could bind (Ohtsubo, 1970).

The minimum number of F tra cistrons was estimated by using genetic complementation tests. Such complementation tests are difficult to perform using two mutants of F' since they cannot stably coexist together because of incompatibility (Datta, 1979). This problem was circumvented by using transfer deficient mutants of

the R-factor R100-1. R100-1, a mutant of R100 derepressed for transfer is compatible with F and is able to complement the transfer defective mutants of F. (Ohtsubo, 1970). Mutants of R100-1 falling into different groups as in the case of Tra- F' mutants were isolated (Ohtsubo, 1970). To establish a partial diploid (diploid with respect to F tra) cell for genetic analysis, R100-1 mutants were transduced by Pl_{kc} to cells already carrying Tra- F' mutants. Genetic complementation leading to restoration of Tra⁺ phenotype and sensitivity to the F' specific phages allowed the identification of at least seven (traA through H, X, Y) cistrons (Ohtsubo et al., 1970). Four of these cistrons (D, E, F and one more) were necessary for the production of pili, mutations in traA showed sensitivity to male specific phages and mutants of traB showed sensitivity to some but not all phages. The order of these cistrons was determined as ABCDEF by genetic complementation of the above mentioned mutants against a series of overlapping deletions generated by Pl transduction (Ohtsubo, 1970).

Achtman et al. (1972) isolated transfer-deficient point mutants of an F'lac element, JCFLO. These mutants fell into three classes on the basis of the phenotypes mentioned above. Genetic complementation was done by the transient heterozygosis technique or by Pl transduction (Achtman et al., 1972). These mutants were obtained by

mutagenesis of a su^- strain carrying $F'lac$ with nitrosoguanidine or ICR-191 (Achtman et al., 1971; Achtman et al., 1972; Willetts and Achtman, 1972). This allowed the isolation of amber or ochre suppressible transfer deficient mutants of $F'lac$. These mutants were of particular value since they not only indicate that the gene encodes for a protein product but also allowed the construction of partial heterozygotes in the su^- strain for genetic complementation tests. P1 transduction was useful in the analysis of non-suppressible mutants (Willetts and Achtman, 1972). Using these two techniques, a total of eleven tra cistrons were identified, traA through traK (Willetts and Achtman, 1972). Of these, mutations in traA, traB, traC, traE, traF, traH, traK, and traJ and some mutations in traG showed resistance to F specific phages and did not produce F pili. Mutations in traD showed production of F pili but were resistant to phage f2. Mutations in traD, traI and some mutations in traG produced F-pili and were sensitive to F specific phages. Mutations in traJ showed pleiotrophic effects in that they were not only deficient in F DNA transfer and F pilus formation but they were also surface exclusion-deficient (Achtman, 1973). Surface exclusion is a property whereby a bacterium carrying F excludes the entry into the same cell of another F plasmid by conjugation. It was therefore proposed that traJ controls

the transcription or translation of traA, traI and perhaps of other tra genes. The six complementation groups described by Ohtsubo *et al.* (1970) as A, B, C, D, E, and F correspond to the cistrons designated traI, traD, traG, traF, traC, and traE respectively of Achtman *et al.* (1972) and Willetts and Achtman (1972). In subsequent studies, ten new genes were identified: traL (Willetts, 1973); traN, traU, traV and traW (Miki *et al.*, 1978); traM (Achtman *et al.*, 1978b); traZ (Everett and Willetts, 1980); traY (McIntire and Willetts, 1980); traT (Achtman *et al.*, 1980) and traQ (Moore *et al.*, 1981a, 1981b).

The twenty-one genes identified so far can be classified into different classes on the basis of phenotypes. The first group comprising fifteen genes, traA, L, E, K, B, P, V, W, C, U, N, F, Q, H, and G, is required for the production of the F pilus and hence for infection by F specific phages. The second group consists of two genes traN and traG which are required for the stabilization of mating pairs. traM, Y, D, I and Z are required for donor conjugal DNA metabolism and forms the third group. traJ, the only gene with regulatory function is in the fourth group. It controls the expression of all genes except traM. Thus, mutations in traJ are deficient in pilus formation and conjugal DNA metabolism. In addition, traJ mutants are good conjugal recipients as they also control the expression of traS and traT, the

genes involved in surface exclusion. traS and traT are not required for the transfer process per se, but as they are located within the transfer region they constitute the fifth group (Achtman et al., 1980).

c) Order of tra genes and direction of transcription.

The order of the newly identified tra genes was derived from genetic complementation tests between representatives of the point mutants in each of the defined cistrons, and a series of deletions (Ippen-Ihler et al., 1972). These deletions were generated by taking advantage of a temperature-sensitive F (Fts) plasmid integrated in the gal operon with λ prophage integrated nearby. After λ induction surviving colonies of such a strain had deletions with one end originating in λ and the other extending to variable ends into the integrated F. Point mutants of traY and traZ are still not available. These two genes were localized on the basis of in vivo cloning experiments (Everett and Willetts, 1980).

In subsequent studies the order of tra genes was determined by the use of bacteriophage Mu-1 insertions (Helmuth and Achtman, 1975). Mu-1 belongs to a special class of genetic elements, called transposons that can insert randomly into the genome of E. coli causing mutations (for a review on Transposable Elements see Kleckner, 1981). Insertion of Mu-1 not only inactivates

the gene in which it is inserted but also the gene or genes downstream to the site of the insertion if the gene is a part of an operon (Bukhari and Zipser, 1972). Insertion into the traA gene abolishes the activity of all genes including traI indicating that traA is transcribed first and that traA-I forms a single large operon of a size of approximately 33 Kb (Achtman and Helmuth, 1975). With the recent addition of two genes, traY and traZ as the first and last gene of this operon this forms a single large traY-traZ operon (Everett and Willetts, 1980); also see Fig. 1 page 16.

Gaffney et al. (1983) utilized both in vivo and in vitro techniques of gene fusion to the promoters of the lacZ gene. These studies showed that traM and traJ are transcribed from their own promoters. Comparison of the level of B-galactosidase revealed that both are transcribed in the same direction as the traY-Z operon. traJ is located between traM and traY-Z. traM and part of traJ have been sequenced (Thompson and Taylor, 1982)

d) Molecular cloning of regions of F-tra.

Segments of the F transfer region have been cloned onto small plasmid vectors or in phage λ by in vitro and in vivo techniques.

in vitro cloning

With the use of restriction enzymes, it is now possible to construct a physical map of a plasmid. If the

Fig. 1. Genetic map of plasmid F. The region of homology between F and R100-1 is shown inside the circle. The arrow shows the location of origin of transfer (oriT) and direction of F transfer. IS3, IS2 and $\gamma\delta$ are insertion sequences. The solid bar represent the Ftra region. The number below the bar corresponds to Kb coordinates of the F physical map. The lines with circles at the end indicate the EcoRI sites. The f numbers represents the EcoRI fragment in their order of mobility in agarose gel. pSH and pRS are the F-EcoRI fragments cloned. The boxes with letters above the solid bar represent the genes and their respective designations. Open lines indicate that the size of the gene is not known. finP, Fertility inhibition gene P. The location of Tra proteins is as follows:

TraJp, TraAp, TraLp, TraKp, TraBp, TraPp, TraTp : outer membrane.

TraEp, TraGp, TraSp : inner membrane

TraMp : inner and outer membrane.

TraVp, TraWp, TraUp, TraNp, TraOp : unknown.

functions carried on each restriction fragment are known, the physical map can be correlated to the genetic map. One way to achieve this is to link the individual fragments to another autonomously replicating plasmid DNA (vector), and determine the function carried by the cloned segments by direct selection of the marker (such as resistance to antibiotic) or indirectly by genetic complementation tests (such as Leu^- to Leu^+). Cloning of DNA segments away from the inc region(s) of the parental plasmid on to a compatible plasmid facilitates the genetic complementation analysis. Since some vectors are present in a large copy number, these techniques could be potentially used also for the amplification of a gene or gene product for further characterization.

EcoRI cleaves F DNA into 19 fragments with the largest fragment being 13.2 Kb and the smallest, 0.18 Kb (Skurray et al., 1976a). F DNA partially digested with EcoRI was cloned into pSC101. pSC101 is a tetracycline resistant (Tc^r) 8.9 Kb plasmid and has a single EcoRI site outside of Tc^r and its maintenance region (Cohen et al., 1973). This allowed the isolation of plasmid derivatives (designated as the pRS series), in which one or more continuous EcoRI fragment of F DNA was cloned into pSC101 (Skurray et al., 1976a; Clark et al., 1976). Single EcoRI fragments from the pRS derivatives were subcloned into RSF2124 (ColE1::Tn3). These were designated as the pSH

series. Introduction of these by DNA mediated transformation into strains carrying representative mutants in tra genes allowed one to locate traJ, A, L, E, B on fragment f6, traC, traF and traH on F1, traD and traI on fragment f2 (see Fig. 1, page 16). Plasmids carrying fragment f1 complemented the non-piliated traG mutants for pilus production but not piliated traG mutants for DNA transfer (Achtman et al., 1978b). Derivatives carrying the adjacent fragment, f17 were able to complement piliated traG mutants for DNA transfer but not the non-piliated mutants. This further confirmed the dual role of traG in pilus production and DNA transfer metabolism and showed that the EcoRI site separated these two functions.

Variability was observed in the complementation by the same fragment when it was cloned in two opposite orientations with respect to the vector. This suggested the possibility of expression from the vector promoter (Skurray et al., 1978). Such expression of tra genes was fortuitous especially in the absence of the traY-Z operon promoter on the fragments of the distal part of the tra operon. Indeed the presence of a promoter on either side of the EcoRI site in pSC101 has been shown (Skurray et al., 1978b).

Although none of the pRS or pSH derivatives were self-transmissible, a derivative carrying EcoRI-f6 was

efficiently mobilized from a strain carrying the conjugative plasmid ColVb (Inc FI). Thus the origin of transfer (oriT), recognized (at least by ColVb) as a site required in cis was localized on f6 and it mapped to the left of traJ (Clark et al., 1976). Subclones of this region which were used to analyse oriT functions are discussed later.

A clone linearized at a HpaI site that cleaves traE and transformed ~~into~~ E. coli cells allowed the isolation of a set of derivatives from a defined point in traE and allowed one to precisely relate traE to traA, traL, traK and traB (Thompson and Achtman, 1979). These studies helped define the promoter proximal end of F-tra.

The promoter distal end contained within the EcoRI fragments f17, f19, and f2 was studied by Manning et al. (1982) and Manning and Morelli (1982). A detailed restriction map of a clone carrying this region was constructed by using deletions and by heteroduplex analysis with the parental DNA. A large number of transposon Tn5 insertions were obtained and mapped using restriction mapping and heteroduplex analysis. This confirmed the order traG, traS, traT, traD and traI that was obtained by genetic techniques. One interesting result was the nonpolarity of Tn5 insertions in some cases. Tn5 is known to cause polar mutations owing to the presence of transcriptional ~~stop~~ signals carried on it

(Berg et al., 1980). Such a non-polar effect of Tn5 was also seen in the promotor proximal region of the F-like R factor, R100 (Hansen et al., 1982). Comparison of the analyses of this region of F and R100 indicated that inspite of overall homology in this region (Sharp et al., 1973; Manning and Morelli, 1982), differences exist with respect to all of the gene products.

BamHI sites flanking the tra region allowed the cloning of the entire F-tra onto a multicopy vector (Johnson and Willetts, 1980). Restriction cleavage maps and genetic analysis of two F-like plasmids R6-5 and R538-1 have been reported (Timmis et al., 1978; Alton and Vapnek, 1978).

in vivo cloning.

Cloning of segments of F-tra onto phage vectors was accomplished by the isolation of λ -tra transducing phages from a lysogen where λ is inserted at the secondary attachment site within or near the tra region (Willetts and McIntire, 1978; Willetts and Maule, 1979, 1980). Transfer-deficient F-tra transducing phages confirmed the previous observation of polarity of mutation downstream to the traA promoter by Mu-1. Some of these mutants showed continued expression of the traI gene in the absence of the traA promoter suggesting the possibility that this gene may be transcribed in part from a promoter located between traD and traI (McIntire and Willetts, 1980). One

of the insertions in the early part of the tra operon produced a mutant which was not complemented by R100 or R1-19 (an F-like R factor). This plasmid-specific tra gene, tray, was located before traA as the first member of the tra operon. Bacteria carrying insertions of λ in the tray gene of F were shown to produce F pili. Thus, tray was concluded to be involved in plasmid DNA metabolism (McIntire and Willetts; 1980).

e) Correlation of genetic and physical map.

Sharp et al. (1972, 1973) analysed the structure of F and F-like plasmids R100 and R1-19 using heteroduplex analysis. It was shown that these plasmids share roughly 50% homology to F and that this homology was in the transfer region. Using the set of tra deletions generated by Ohtsubo et al. (1970), they localized traA-E genes between position 64.5 to 94.5 Kb. Subsequent cloning of F DNA fragments allowed them to precisely calculate the sizes of smaller fragments and thus to refine the correlation of the genetic and physical map of F (see Fig. 1). One third of the F DNA is required for conjugal transfer. Although not shown on this map several other functions such as replication and incompatibility and insertion sequences have been identified and assigned to the remaining two-third of F DNA. The genes for pilus production are clustered in F-tra and located near the

origin of transfer. The genes required for donor conjugal DNA metabolism are more scattered.

f) tra gene products.

A comprehensive analysis of tra gene products was begun by Kennedy et al. (1977). Use of the plasmid chimeras constructed in vitro allowed the identification of various tra proteins in minicells and in vitro. Minicells are small aberrant bodies produced by a mutant E. coli strain and are devoid of genetic material. Minicells into which a plasmid is introduced can be used as a protein synthesizing system (for a review see Frazer and Curtiss, 1975). Since chimeric plasmids carried only a part of tra, unambiguous assignments of tra proteins to a specific region on plasmid DNA was possible. To confirm this, the plasmid chimeras were mutagenized in vitro by hydroxylamine (Humphrey et al., 1976) to obtain mutants in a defined gene (Kennedy et al., 1977). For those genes where such mutants were not available, defined point mutants of F'lac isolated previously (Achtman et al., 1971) were moved to plasmid chimeras by genetic techniques. Such mutations led to the absence of only one particular protein. In addition, proteins can be identified for which no known mutants are available, thus defining a new gene. The results of experiments using such minicells and of in vitro experiments were consistent and correlated well with the loss or alteration of protein

products. This allowed the identification and assignment of proteins for genes recognized at that time TraMp, TraJp, TraAp, TraLp, TraEp, TraBp, TraCp, TraFp, TraHp, TraSp, TraTp, and TraDp. The estimation of the molecular weight of these proteins also allowed one to infer the respective sizes of the genes. They are indicated by boxes in Fig 1. In addition, three major proteins in the tra region were identified (and designated 6c, 6e and 2a) which could not be assigned to any of the known genes (Kennedy et al., 1977). Subsequently the genes for these were identified as traY (TraYp), traP (TraPp) and traZ (TraZp). Since these proteins were identified in a minicell system, their intracellular location was determined. The majority of the proteins involved in conjugation are strongly associated with the membrane. TraJp, TraAp, TraLp, TraKp, TraBp, TraPp, and TraTp are outer membrane proteins; TraEp and TraGp, TraMp, TraSp are inner membrane proteins (Kennedy et al., 1977; Achtman et al., 1979). Interestingly, the TraMp protein has been found to be associated with both inner and outer membranes (Achtman et al., 1979). The tra proteins synthesized both in vivo and in vitro showed identical MW indicating that processing is not necessary for their incorporation into the cell membrane, as is known to be the case for some proteins exported to the periplasm and outer membrane (Achtman et al., 1979). Products of traV, traW, traU,

traN, and traQ have not yet been identified. TraGp (product of traG) has recently been identified as a cell envelope inner membrane protein (Willetts and Maule, 1980; Manning *et al.*, 1981).

g) F-pilus biochemistry

As discussed earlier, a total of at least fifteen genes traA, L, E, K, B, P, V, W, C, U, N, F, Q, G, and J have been identified which are required for the production of functional F pili. The product of these genes are interchangeable between F and IncFII plasmids, indicating an overall similarity of the pili produced by these plasmids. However, the 2 groups of pili can be differentiated serologically (Lawn and Meynell, 1970). Purified F pili are assembled from one primary subunit, F pilin, which has a MW of 12,000 daltons (Brinton, 1971; Beard *et al.*, 1972).

Mutations in gene traL through traG are shown to accumulate F pilin in the inner membrane suggesting the possibility that they may be involved in modification, assembly, extrusion and possibly retraction of F pili (Moore *et al.*, 1981a, b). The evidence that traA is the structural gene for F pilus is overwhelming and at least one additional tra gene, traQ, is needed for its activity (Moore *et al.*, 1981a, b). Minkley *et al.* (1976) showed that F pilin purified from a supF strain carrying an amber suppressible mutation in traA showed an additional

tyrosine residue. Mutation in traA have been isolated that affect the binding of RNA phages but which are still transfer proficient (Willetts et al., 1980). The product of this gene; TraAp has a MW of 14,000. It does not comigrate with purified F pilin nor react with F pilin antiserum (Manning and Achtman, 1979). Using λ tra transducing phages carrying traA it was shown that a 14,000 dalton protein accumulated in the inner membrane of cells. However, this polypeptide was replaced by a 7,000 dalton polypeptide in the presence of traQ (Ippen-Ihler et al., 1984). It was suggested that the traA product is processed to F pilin in a traQ dependent reaction. However it's possible regulatory role has not been ruled out.

h) Regulation of transfer.

Most of the naturally occurring conjugative plasmids show only a fraction of their full potential for transfer (0.1-1%), pilus production and surface exclusion (Meynell et al., 1968; Watanabe and Fukasawa, 1962).

This phenomenon called Fin⁺ (Fertility inhibition) is due to the inhibition of transfer by certain gene products. F transfer is naturally derepressed and expresses its full transfer potential. The introduction of an F-like plasmid (e.g. R100) into the F⁺ cells results in the reduction of transfer associated functions of F (Willetts and Finnegan, 1970). Derepressed mutants of R100 have been isolated

that transfer F (and R100) at a frequency comparable to that of F from an R- cell. Two structural genes of R100 which are required for the inhibition have been identified (Finnegan and Willetts, 1971; 1973). These are designated as finO and finP (previously called traP) and together they form the FinOP system. One of the genes, finP, is located between traJ and oriT (of F and R100). F is a naturally occurring finO- mutant and susceptible to transfer, inhibition by the finO product of F-like plasmids. Using λ tra transducing phages it was shown that FinOP represses the transcription of traJ which in itself is a positive regulator for the traY-A operon. Fig. 2 (page 27) illustrates this regulatory circuit. Having reviewed the genetic and functional organization of genes constituting the F-tra system, most of the rest of this review will be concerned with the process of F mediated conjugation.

II) The process of conjugation

a) Cell-to-cell interaction.

The requirement of co-ordinated expression of twenty two or so genes discussed above indicates the complexity of the conjugation process. A model has been proposed that summarizes the events occurring at the cellular and subcellular level (Willetts and Skurray, 1980; Manning and Achtman, 1979). In a mixed population of donor and recipient, the pilus produced by the donor

Fig. 2. Regulatory circuit of F transfer. The solid bar with letter below it represents Ftra. The horizontal arrow above the bar represent the direction of transcription of the traM, finP, traJ and traY-Z operons. In addition traI-Z is transcribed independent of the traY-Z. The product of finP of R and F are represented as FinP_R and FinP_F. The site of action of FinOP_F is shown by an arrow. J* indicates the site of action of the positive regulator of the traY-Z operon. ? indicates uncertainty of the regulation by traJ.

cells allow them to recognize and bind to the recipient cells. This subsequently forms stable mating pairs (or aggregates) which are resistant to mild shear forces. Such specific contact triggers a signal that prepares the plasmid DNA for transfer. Transfer originates at a specific site, oriT. A specific strand (which is nicked at or near oriT) is transferred to the recipient cell. A complementary copy of the transferred DNA strand is synthesized in the recipient and the strand transferred is synthesized in the donor cell. Recircularization of DNA strands in donor and recipients, a process called replication, allows plasmids to stably establish themselves and express. The mating aggregates now disengage and both the donor and recipient can then act as donors in another round of mating. This process of conjugation is visualized as a multistep event. These steps are discussed below, in the order that they may be occurring.

b) Sex Pili (Pili).

The study of conjugation was stimulated by the discovery of surface organelles on F and Hfr cells that were absent on F- cells (Brinton et al., 1964; Brinton, 1965). These organelles called F pilus were implicated to be involved in plasmid transfer (Brinton, 1965). Since then, conjugative pili have been observed on cells carrying representative plasmids of several Inc groups

(Bradley, 1980b). These pili can be classified into 3 groups on the basis of morphological and serological characteristics; thin flexible (e.g. IncL_u plasmids), thick flexible (as in F) and thin rigid (e.g. IncN plasmids). This classification correlates partially with the classification based on incompatibility (Bradley, 1980a).

F pili have been recognized and distinguished from the fimbriae and flagella of E. coli and other Gram negative bacteria by their ability to adsorb male or pilus specific RNA bacteriophages (f2, M12, R17, MS2 or QB) along their sides (Crawford and Gestland, 1964; Brinton et al., 1964) and DNA bacteriophages (f1, fd, M13) which bind to their tips (Caro and Schnos, 1966). One to three pili are produced on the cell surface depending on the growth conditions (Curtiss et al., 1969). When donor and recipient cells are mixed in a liquid medium, specific contact results from the interaction of donor F-pili and an unknown receptor on the surface of the recipient. Active pili are necessary for plasmid transfer. There are mutants which produce pili but which are temperature sensitive for conjugation after incubation for some time at restrictive temperature. Brinton (1965) and Novotny et al. (1969) showed that removal of pili by blending resulted in the loss of donor ability. Harden and Meynell (1972) showed that binding of F-antibody prevented the

transfer. RNA phages which adsorb to sides of the pilus also prevent transfer. That the tip of the pili may be involved in some manner was shown by the observation that the addition of DNA bacteriophages (Ou, 1973), and Zn^{+2} ions (Ou and Anderson, 1972) which adsorb to the tip of pili prevent DNA transfer. At low SDS concentration, F pili are dissolved from the cell surface and this also prevents mating aggregate formation and DNA transfer (Achtman et al., 1978).

c) Role of pilus in transfer.

Although it is clear that the pili are necessary for transfer, their exact role in conjugation is not clear. It was observed by light and electron microscopy that mating pairs are formed by an invisible bridge assumed to be the F pilus (Brinton, 1965). Ou and Anderson (1970) found that two kinds of mating pairs are formed, one that lead to a loose connection and another that showed cell-to-cell contact. Using a micromanipulator technique, they were able to separate these two kinds of mating pairs and show that recombinant formation was significantly higher from cells that were in close mating pairs than from cells that were in loose mating pairs. They suggested that the role of F pili is to establish an initial contact between mating pairs, and to bring mating pairs into such a close proximity that this increases the transfer frequency. However, such

close contact has not only been observed with the mating pairs but also between pairs of donors (Brinton, 1971) and pairs of recipients (Lancaster and Skvarla, 1970).

Three models have been proposed for the role of pili in DNA transfer during conjugation. Optical and X-ray diffraction studies on F pilus have suggested that it is a hollow cylinder with a 20 Å axial hole. (Helmuth and Achtman, 1978; Folkhard et al., 1969). Negative staining with bacteriophages led Brinton (1965) to propose a "F pili conduction" model which considers that the DNA is transported across the cells through this axial hole into the recipient. A slightly different version of this model, also proposed by Brinton (1971) as the "conveyer belt" model, is based on the concept that F pili are two parallel chains. One strand of plasmid DNA to be transferred is attached to one chain and the two pilus strands move with respect to each other as a conveyer belt delivering the DNA strand into the recipient. However, to date the sex pilus-DNA complex has not been isolated and these models remain speculative. An indirect evidence against both models is that SDS which dissolves pili thereby preventing mating pair formation is unable to prevent DNA transfer once such mating pairs are formed (Achtman et al., 1978).

A third model considers that once the initial contact with recipient cells is made, the pilus retracts

bringing cell wall-to-cell wall contact (Novotny and Fives-Taylor, 1974, Fives-Taylor, 1978). This model is based on the observations of Marvin and Hohn (1969) and Jacobson (1972) for phage infection. It was observed that F pili progressively disappear with time after infection with filamentous bacteriophages. This was interpreted to mean that pili retract during infection and eventually only the tip of the pilus is exposed on the cell surface since bacteriophage appeared to be adsorbed directly to the cell surface. Various conditions are known which make F pili disappear. These are incubation in the presence of NaCN (Novotny and Fives-Taylor, 1974), arsenate (O'Callaghan et al., 1973) and shift of temperature from 37° C to 25° C or to 50° C (Novotny and Lavin, 1971). Disappeared pili do not appear as free pili in the supernatant nor are these conditions known to degrade free pili. This was interpreted as evidence for retraction. Consistent with the pilus retraction model, a mutant which is pilated, transfer deficient and defective in pilus retraction was isolated (Burke et al., 1979). Although the mutation is not precisely mapped it was concluded that traD, traG and traI are not involved in pilus retraction (Fives-Taylor, 1978).

d) Formation and stabilization
of mating aggregates.

Donor F pili recognize the recipient cells and bind to them. This does not however necessarily lead to successful conjugal transfer. Such specific pairs can form between two dead cells (Falkow, 1975). The initial mating aggregates are then converted to a more stable form which is resistant to mild shear forces such as pipeting and centrifugation (Achtman, 1975). The size distribution and proportion of donor cells in a stable mating aggregates can be varied and depends on the initial input ratio of donor to recipient (Achtman, 1975). The cell-to-cell contact may be actively converted to cell wall-to-cell wall contact which is more common and also more efficient in DNA transfer (Ou and Anderson, 1970). That such "real" contact may occur was indicated by some exchange of outer membrane proteins between mating cells (Goldschmidt and Curtiss, cited in Achtman and Skurray, 1977). Using the Coulter counter technique (Achtman et al., 1978b) it was possible to distinguish between the unstable and stable mating aggregates. Manning et al. (1981) showed that neither DNA transfer nor triggering of conjugal DNA synthesis (products of traM, traD, and traI) are necessary to form stable mating aggregates. However, pilated traG mutants and traN mutants did not efficiently accumulate stable mating aggregates. Moreover, many more

morphologically similar mating aggregates were formed than those that succeeded in F DNA transfer (Manning et al., 1981). This study therefore indicated that stabilization of mating pairs requires the product of traG and traN and is distinct from triggering of the DNA metabolism and conjugal DNA replication. Part of traG is required for pilus production. The strategic location of TraGp in the inner membrane suggests that it is a part of the F pilus which spans the inner and outer membranes allowing it to be involved in both pilus synthesis as well as in converting the initial contact to stable mating aggregates. In light of the pilus retraction model, it is conceivable that pilus retraction leads to wall-to-wall contact for stabilization and that the traG product plays a dual role in these stages. In these events, the role of the recipient has not been discussed. This has been reviewed by Willetts and Skurray (1980) and by Manning and Achtman (1979).

e) Conjugal DNA metabolism.

Subsequent to cellular contact, the process of transfer of DNA in conjugation can be separated in three different stages as i) processing of DNA in donor ii) transit between the donor and recipient and iii) entry into the recipient followed by stable inheritance of the plasmid (Clark and Warren, 1979). These steps are initiated in response to a mating signal (Ou, 1978). Such

a signal is generated when donor organelles, probably pili, interact with the appropriate recipient receptor molecule (Ou and Reim, 1978). The recipient molecules generating this signal and the nature of this signal have not yet been identified. Ou and Yura (1982) isolated two types of donor mutants. In type I mutants donor conjugal DNA synthesis (DCDS) could be induced but transconjugants were formed poorly due to a change in the inner membrane; in type II the mating signal for DCDS was not generated, due to a change in the outer membrane. Transfer replication is triggered during mating since it does not occur in the absence of recipient cells (Bresler et al., 1968; Sarathy and Siddiqi, 1973; Wilkins and Hollow, 1974). The addition of minicells (as recipients) triggers DNA transfer replication (Ou, 1975; Fenwick and Curtiss, 1973); however, it is not necessarily accompanied by DNA transfer. Thus conjugal DNA replication and DNA transfer, although normally coupled, are not dependent on each other (Achtman and Skurray, 1977). The mating signal also does not appear to be necessary for nicking at the oriT site (Everett and Willetts, 1980) and the plasmid exists in covalently closed circular (CCC) form and nicked relaxed form. In the donor, the mating signal may be due to a change in either a location of a protein or to an allosteric change in its structure which may activate transfer from oriT. The product of traM (TraMp) is not

required for pilus synthesis, mating pair formation or nicking at oriT but is required for transfer of DNA

(Everett and Willetts, 1980). TraMp is located in the inner membrane (Kennedy et al., 1977) and can bind to a region containing oriT sequences (Musgrave and Achtman, cited in Thompson and Taylor, 1982). These properties would be suitable for such a triggering role. The sequence of traM (Thompson and Taylor, 1982) may aid in understanding its role in conjugation.

f) Origin of transfer and
nicking of DNA at the origin.

Transfer of the plasmid molecule originates at a specific site located on plasmid DNA which is designated as oriT (origin of transfer). It was identified as a cis-dominant transfer defect (Willetts, 1972; Guyer and Clark, 1976; Guyer et al., 1977). It is located at one end of tra immediately next to the tra control region. The oriT site is central to the transfer process and includes the nick site and associated binding site(s) for the nicking proteins. Plasmids containing such sequences can be identified via efficient transfer (often called mobilization) from bacteria carrying a compatible plasmid that can provide necessary trans-acting functions. Such transfer by donation is distinct from the transfer via cointegrate formation of a conjugative and non-conjugative plasmid, termed conduction, mediated by Insertion

sequences (Clark and Warren, 1979). In vitro cloning techniques have been used to isolate and analyse the oriT containing segments of F. Subcloning the EcoRI-oriT clone (Achtman et al., 1978c) has reduced the region containing functional oriT progressively from 1.1 Kb (Thompson and Achtman, 1978), to 540 base pairs (bp) (Johnson et al., 1981) and then to 373 bp (Everett and Willetts, 1982; Thompson et al., 1984).

Conjugal DNA metabolism is initiated at a nick in a specific plasmid DNA strand at oriT (Willetts, 1972; Reeves and Willetts, 1974). Everett and Willetts (1980) developed a novel in vivo system to characterize the oriT of F. When oriT phage constructed in vivo or in vitro was infected on cells carrying F'lac, 10% of the phage genome showed a nick on the "transferred" (the strand which is normally transferred to the recipient) DNA strand. This nicking was plasmid specific and F⁻ cells did not show any nicking. The presence of a 3'OH terminus nonsusceptible to ligation by T4 DNA-ligase indicated that after nicking the 5' end may be modified in some way (Everett and Willetts, 1980, Thompson et al., 1984). Similar nick sites have also been located on plasmids of other Inc groups; ColE1 (Bastia, 1978); RK2, pSC101 (Nordheim et al., 1980). Using various point mutants and deletion mutants, the product of two tra genes (traY, traZ) were identified as being involved in nicking at the

oriT. The product of traJ was also required for nicking. However, this may be due solely to its regulatory function. It is surprising that no point mutants in these two genes, specially of traZ because of its size (2 Kb) were isolated in previous studies by Ohtsubo et al. (1970), Achtman et al. (1971, 1972) or Miki et al. (1978). The location of traY and traZ as the first and last gene of the traY-Z operon indicate that they are at least 30 Kb apart (see Fig. 1). The TraYp and TraZp were localized in the cell envelope and cytoplasm respectively (Manning and Achtman, 1979; Achtman et al., 1979). This location may allow them to form a complex with the nicked strand of oriT at the cell surface. No endonuclease activity has been found to be associated with TraYp and TraZp in vitro (Everett and Willetts, cited in Willetts and Skurray, 1980).

g) Mobilization of non-conjugative plasmids and specificity of oriT

Some plasmids such as ColE1 and CloDF13 are unable to promote their own transfer and are non-conjugative. Nevertheless, in the presence of many unrelated conjugative plasmids such as F and R64-11 they are able to transmit themselves at frequencies comparable to that of the cohabiting conjugative plasmids, a process termed mobilization (Clark and Warren, 1979; Reeves and Willetts, 1974; Warren et al., 1978). Such mobilization does not

seem to occur by physical association of the two plasmids. It requires both a region of DNA in cis (Warren et al., 1978) and proteins encoded by the mobilizeable plasmid (Dougan and Sherratt, 1977; Warren and Sherratt, 1977). This region in cis has been termed bom (basis of mobility) (Warren et al., 1978). Deletion and transposon insertion mutants have been isolated which are not mobilized by conjugative plasmids and they are termed mobilization deficient (Mob-) (Hershfield et al., 1976; Dougan and Sherratt, 1977). These mutants were unable to form a plasmid DNA-protein relaxation complex, a complex the formation of which is associated with mobilization (Inselburg and Ware, 1977; Helinski et al., 1974). When treated with agents such as protease or ethidium bromide, the relaxation complex can introduce a nick into one of the DNA strands producing a 3' terminus which is suitable as a primer for DNA synthesis and a 5' terminus covalently bound to a protein. There are three proteins of 60,000; 16,000; and 11,000 daltons associated with this complex. The isolation of mob recessive mutations indicated that at least one protein (the 16,000 dalton protein) is encoded by mob (Lovett and Helinski, 1975; Dougan and Sherratt, 1977). bom is located very close to or may be identical with nic, the site at which the ColE1 is nicked by the protein(s) of the relaxation complex (Warren et al., 1978; Inselburg and Applebaum, 1978). It is very likely that if

ColE1 has a unique nic site that this relaxation nick is in fact oriT. Although the presence of a conjugative plasmid in the donor is necessary, its transfer to the recipient is not. All the F tra genes except traM, I and Z are required for ColE1 mobilization (Alfaro and Willetts, 1972; Willetts and Maule, 1979; Everett and Willetts, 1980). The product of F traD is required for mobilization of ColE1 but not for the mobilization of CloDF13 (Van de Pol *et al.*, 1978). A mutant in traG that is able to produce pili but does not form stable mating pairs is unable to mobilize CloDF13. Therefore, CloDF13 can not form stable mating pairs.

oriT sequences of ColE1, CloDF13 and other plasmids such as pSC101, RSF1010, RK2 have been determined which allow the direct comparison of the respective oriT at the DNA sequence level (Oka *et al.*, 1979; Bastia, 1978; Guiney and Jakobson, 1983; Willetts and Wilkins, 1984). The oriT sequences of non-conjugative but mobilizeable plasmids differ from each other and also from those of conjugative plasmids that are able to mobilize them (e.g. F and ColE1, RK2 and RSF1010). This can be also seen from the non-requirement of the traYZ nicking proteins in the mobilization of ColE1 and CloDF13 (Everett and Willetts, 1980). Even among the IncF plasmids which code for genetically similar tra systems, three oriT sequences have been determined (Willetts and Wilkins, 1984). Thus the

oriT sequences differ from plasmid to plasmid and can be more specific than the overall tra system. An additional specificity can be recognized by the ability of some conjugative plasmids to mobilize a non-conjugative plasmid at a high efficiency but others only poorly. Thus F tra pilated mutants that are able to form stable mating aggregates are not mobilized by conjugative I_a plasmid (Willetts, 1970). Such specificity must therefore be recognized by mobilization of a non-conjugative plasmid by a conjugative plasmid.

h) Transfer of DNA.

Once the mating signal is generated, the nicking at oriT is followed by the transfer of F DNA to the recipient in a definite sequence. When F is integrated into the host genome then part (or rarely all) of the chromosome is also transferred. Nicking of F DNA at oriT allows transfer to proceed in such a manner that the tra genes are transferred last. Curtiss (1969) used minicells as recipients in crosses with F, Hfr and F'. Due to their small size these minicells can be separated from normal E. coli cells. When F' and F was used as donors the minicells were found to contain both single stranded (ss) and double stranded (ds) DNA. With the Hfr strain virtually all minicells were found to contain ss DNA. To determine which of the two strands is transferred, Ihler and Rupp (1969) made use of an Hfr λ^+ lysogenic strain.

Their technique was based on the observation that two strands of the λ genome contain different A+T/G+C ratio and such strands could be separated on polyuridylylate-polyguanylate (poly(U,G))-CsCl gradients (Hradeana and Szybalski, 1967). After mating, the selectively labeled Hfr⁺ λ strain with unlabeled F⁻ λ recipients in unlabeled medium, the λ DNA was isolated from the recipient cells after zygotic induction (Ihler and Rupp, 1969). It was the denser strand (on polyUG-CsCl gradient) of the two that was transferred with the 5' end first, leaving behind the lighter strand in the donor. This confirmed the previous and similar observations made by other workers (Ohki and Tomizawa, 1968; Rupp and Ihler, 1968). In the case of F, the "transferred" ss in the recipient and the ss left behind in the donor are both used as templates to convert them to ds DNA (Vapnek and Rupp, 1969). Thus, after mating, the F DNA can be recovered from donor and recipient in a CCC form. R358-1 and R64drd-11, IncFII and IncI_q plasmids respectively, also show similar transfer of the denser strand during conjugation (Vapnek et al., 1971; Vapnek and Rupp, 1971).

For transfer of a DNA strand to occur the duplex plasmid DNA must unwind subsequent to the introduction of a nick. Four DNA helicases, the enzymes that are capable of unwinding ds DNA in an ATP dependent reaction have been recognized (Kornberg, 1980). One of these helicase I, was

found only in the F^+ strains and not in F^- strains (Abdel-Monem et al., 1983). The transfer of the F factor to recipient cells by conjugation permits the purification of helicase I from the transconjugant cells. Using the EcoRI clones of F tra, it was found to be identical to the product of traI gene, TraIp (MW 180,000 dalton). Helicase I requires a ssDNA region of 200 nucleotides for the start of unwinding and it can not start at a nick (Kuhn et al., 1978). It was proposed that the traYZ-induced nick is extended probably by the traM protein to allow desirable ssDNA to form (Abdel-Monem et al., 1983). Since helicase I unwinds DNA in a 5' to 3' direction by moving along the strand to which it is bound, the enzyme must act on the "transferred" strand. Another enzyme, DNA gyrase, encoded by host E. coli, has also been implicated to be involved in conjugal DNA transfer. Nalidixic acid inhibits the transfer of plasmids from sensitive donor cells (Barbour, 1967; Cohen et al., 1968). Nalidixic acid acts on one of the subunits of DNA gyrase (Higgins et al., 1978). DNA gyrase may be required for DNA supercoiling or to separate strands during transfer. The observation that the DNA strand already initiated for transfer, completes transfer in the presence of nalidixic acid, supports the latter possibility without excluding the former (Bouck and Adelberg, 1970). The product of traD is required for transfer. In its absence there is a reduced rate of donor

conjugal DNA synthesis. Since it is not required for stabilization of mating pairs it has been suggested that its role is to aid DNA unwinding or transport (Kingsman and Willetts, 1978).

i) DNA synthesis for conjugal DNA transfer.

As stated earlier, a specific strand of DNA is transferred and both the newly transferred DNA strand and the one that is left in the donor are converted to CCC ds DNA. These are termed RCDS (recipient conjugal DNA synthesis) and DCDS (donor conjugal DNA synthesis) (Willetts, 1981). Studies have been carried out to determine if the enzymes normally required in replicative DNA synthesis are involved or to ascertain if transfer can proceed in the absence of all DNA synthesis. Bonhoeffer et al. (1967) observed that DNA synthesis in the donor is not required for genetic transfer since the frequency of recombinant formation was normal with a ts dnaB Hfr donor and when mating was done at the non-permissive temperature dnaB(ts) mutants do not replicate DNA at a non-permissive temperature (Wechsler and Gross, 1971). Similar observations have been made by other workers (Bresler et al., 1968, 1973; Vapnek and Rupp, 1971; Wilkins and Hollom, 1974). Marinus and Adelberg (1970) have shown that dnaA, dnaC, dnaE and dnaG do not play any role in transfer. An enhancing or diminishing effect on the thermoresistance of dnaB mutants by a variety of

conjugative plasmids has led to the suggestion that plasmids may carry dnaB analogue (ban) genes which may act in conjugation (Wang and Iyer, 1977, 1978). Kingsman and Willetts (1978) showed that DCDS requires untranslated RNA and is carried out by DNA polymerase III. If DCDS is envisioned to occur by a rolling circle model, a protein similar to single stranded DNA binding protein (ssb), destabilizing ds DNA, could be required for DNA replication. A region, ssf, encoding such a protein has been localized on F DNA that is transferred early during conjugation (Kolodkin et al., 1983). Its direct involvement in conjugal DNA replication has not been shown.

In the recipient, transfer of ss DNA is associated with the synthesis of the complementary DNA strand and circularization of the plasmid to CCC DNA form. Wilkins and Hollom (1974) have shown that the replication of the ss DNA in the recipient is independent of dnaB and requires DNA polymerase III. Since the 5' end is the leading end of the "transferred" strand, it is expected that RCDS will be discontinuous. This is inferred from a synthesis of RNA primer in a process involving dnaB product (McMachen et al., 1977). Rifampicin treated recipient cells can form duplex-DNA indicating that the expression of the transferred strand is not needed for RCDS and that host enzymes may carry out replication and

recircularization (Hiraga and Saitoh, 1975). However, the possibility that plasmid encoded proteins from the donor may be transported together with DNA during conjugation has not been ruled out (Kingsman and Willetts, 1978).

Recircularization may occur by a process analogous to that described for ϕ X174 (Eisenberg *et al.*, 1977). In this case, the 5' and probably 3' end of the transferred DNA strand may form a complex with membrane proteins at a cell-to-cell junction. Ligation of 3' to 5' end would generate an open circular plasmid DNA configuration that can be converted to supercoiled form after completion of DNA synthesis.

Once the DNA transfer is completed the donor and recipient actively segregate. This may be dependent on the entire transfer of the plasmid since in Hfr x F- cross ~~such~~ separation was not seen. Alternatively, expression of plasmids in donor and/or recipient may be required.

j) Barriers to conjugation.

F plasmids not only confer conjugal ability to the host cells, but also render them into poor conjugational recipients, a phenomenon called entry exclusion or surface exclusion (Lederberg, *et al.*, 1952). It was suggested that a decrease in mating aggregate formation is the result of the surface exclusion of an incoming isogenic plasmid by F (Achtman *et al.*, 1971). However, when an Hfr strain was used as donor on recipients with or without F

plasmid it did show reduction in the recombination frequency with the F^+ recipient but not in mating aggregate formation. This directly contradicts the above suggestion (Eckerson and Reynard, 1977). Some physiological conditions such as growth to late stationary phase are known to convert F^+ cells to accept isogenic plasmids at a frequency comparable to F^- cells. This state of F^+ is known as F^- phenocopy (Lederberg et al., 1952, Willetts and Maule, 1974). Using a series of *tra* deletions within *F* *tra* genes, *traS* was mapped between *traG* and *traD*. Polarity on the expression of *traS* by Mu-1 insertion in *traG* suggested the order as *traG* *traS* *traD* and that it is a member of *traY-Z* operon (Helmuth and Achtman, 1975). In vitro mutagenesis of a cloned EcoRI-segment carrying this region showed that there are two genes, *traS* and *traT* that are required for exclusion (Achtman et al., 1980). These mutations do not affect pili production and leaky mutants do not affect conjugal transfer. The reduction in frequency of transfer in an F^+ X F^+ cross compared with F^+ X F^- cross was 15 to 50 fold (Achtman et al., 1980). The location of the product of *traS* (TraSp, MW 25,000 dalton) in the cell envelope correlates with its role (Achtman et al., 1977, Kennedy et al., 1977). Beard and Bishop (1975) suggested that the intact peptidoglycan layer is essential for surface exclusion. Their results showed that in F^+ X F^+ crosses

when spheroplasts of active F^+ cells were used as recipients, they did not show any surface exclusion whereas intact F^+ cells used as recipients with spheroplasts of F^+ as donors did show surface exclusion. A number of surface exclusion groups have been recognized in the IncF complex (Alfaro and Willetts, 1972).

Surface exclusion genes have been implicated to be involved in the conjugation-related phenomenon of lethal zygosis (Gross, 1963). When an excess of Hfr cells is mixed with F^- cells the recipient bacteria are killed. However, in $F^+ \times F^-$ cross the recipient cells are not killed (immunity to lethal zygosis, ilz) (Skurray and Reeves, 1973; Skurray and Reeves, 1974). Lethal zygosis may be due to the leakage of cytoplasmic material during conjugation. $F^+ \times F^-$ crosses should also be able to show lethal zygosis and indeed at a high ratio of F^+ to F^- cells (200:1) lethal zygosis was seen (Ou, 1980). Skurray and Reeves (1974) suggested that the ilz is due to the transfer of ilz genes on F in F^+ to F^- mating and since ilz is located within the tra region which is transferred last in Hfr $\times F^-$ crosses, ilz was not seen with Hfr strains. There are two ilz genes, that are recognized one of which, ilzA may be identical with surface exclusion genes traS and traT, whereas the other is located outside tra (Skurray et al., 1976).

The P-transfer system.

Plasmids belonging to the IncP group can transfer to and among many bacterial species within and outside Enterobacteriaceae. It has been reported that this group of plasmids can be transferred to and maintained by all Gram negative bacterial species in which this has been tried (Datta and Hedges, 1972; Olsen and Shipley, 1973; Barth, 1979; Thomas, 1981). Two views have been presented for the mechanism underlying their promiscuous nature. One view is that this may be due to replication and maintenance in a wide variety of bacteria. The other considers it to be a manifestation of a novel transfer system. The extensively studied members of this group, R68, RP1, RP4, RK2 are very similar if not identical (Burkardt et al., 1979).

The P conjugative system differs from the well-studied F conjugation system in the following respects: 1). Bacteria carrying these plasmids produce P-pili which are short and pointed and are morphologically distinct from F pili (Bradley, 1974). A set of bacteriophages different from those that adsorb to F pili adsorb to P-pili. These are the filamentous DNA phage P13 which adsorbs probably to the tip although such an attachment has not been demonstrated (Stanisich, 1974); RNA phage PRR1 (Olsen and Shipley, 1973) which binds to the sides of the pili (Bradley, 1979); and bacteriophages PRD1

selective media without recipient served as the controls. If killing of Klebsiella pneumoniae was to be tested simultaneously, then the last selective plate was minimal glucose media spotted and spread with 0.2 ml of 10^8 cfu/ml of K. pneumoniae.

Sensitivity of strains to phages IKE and PRD1 was determined by a spot test (Konarska-Kozłowska and Iyer, 1981b). 0.1 ml of an overnight culture to be tested was spread on a PA plate and dried. 0.05 ml of a high titre of phage suspension (10^{11} pfu/ml) was spotted and air-dried. An area of clearance at the spot was observed in those instances where the culture was phage-sensitive.

Procedures involving the use of bacteriophages.

Phage lysate of IKE and PRD1.

High titres of phages were prepared as follows. 0.1 ml of phage dilutions were mixed with 0.2 ml of an E. coli strain carrying pCU1 for IKE and Salmonella typhimurium LT2 carrying RP1 for PRD1; 2.5 ml of molten R-top agar was mixed and poured on PA and incubated at 37°C for 15-18 h. Using a sterile glass slide, the R-top agar from plates that showed confluent lysis was collected in a sterile tube. It was vortexed thoroughly and then spun down at 27,000 Xg for 25 min. The supernate was transferred to a sterile tube. This procedure yielded 10^{10} - 10^{11} plaque forming units per ml.

of sensitivity to one or more of the P pilus-specific phages and loss of surface exclusion. Physical mapping of Tn7 insertion mutations led to the identification of two regions flanking the Km resistance gene of RP4 designated as tral and tra2. Using the P1 transductional complementation analysis, a minimum of five complementation groups were identified. Some mutants that were able to adsorb PRR1 showed marked increase in the number of pili. This led these workers to suggest that P-pili are retractable and that in these mutants they were locked in the extended position. Subsequently, an additional tra region was identified, tra3, away from tral and tra2 (Barth, 1979). This was identified on the basis of the loss of conjugal ability and surface exclusion. This component of the P-tra system has been mapped to three distinct genetic loci at 18-19 Kb, 24-25 Kb and 23-24 Kb coordinates on the PR4 map on the basis of the various extents to which surface exclusion is lost (P. Barth cited in Thomas, 1981). Watson et al. (1980) and Schmidt et al. (1980) have cloned tral of RP1 on to a plasmid vector compatible with RP1. This allowed the construction of stable partial heterozygotes of the Tra-derivatives of RP1 and the Tra1 clone. The genetic complementation analysis has shown that there are at least four tra genes located on the Tra1 region of RP1.

RK2 can be isolated as a supercoiled DNA-protein

relaxation complex (rlx) and the nick introduced by treatment with agents such as proteases has been mapped at 47-48 Kb co-ordinates on the RK2 map by Guiney and Helinski (1979). A cis-acting region for transfer, presumably oriT, also maps in this region. Nucleotide sequence analysis has revealed that a 760 base pairs (bp) region contains oriT and rlx (Guiney and Jakobson, 1983). A fragment of 112 bp carrying the functional oriT has been cloned. Comparison of the mobilization frequency of a clone carrying the 760 bp fragment by several IncP group plasmids has revealed that there are two subgroups P_a and P_β among IncP plasmids (Jakobson and Guiney, 1983). The oriT carrying plasmid of one group is mobilized at least 1000-fold less efficiently by a plasmid from another group compared to mobilization by a plasmid within the same group. Using a λattRP4 derivative, an Hfr derivative of E. coli was shown to transfer chromosomal markers in a polarized manner (Watson and Scaife, 1980). On the basis of this data and the analysis of λattRP4 deletion derivative, it was concluded that like F the transfer genes of this plasmid enter the recipient last (Al-Doori et al., 1982).

Figure 3 (page 53) shows the organization of the P-tra system. Combined data from all the analyses show that P-tra is approximately 19 Kb in size and located at three distinct regions on the plasmid. R18 is an IncP

Fig. 3. Map of RP4/RK2/RP1 showing the locations of Tra regions (for reference see text). oriT, origin of transfer; rlx, DNA-protein relaxation complex; sex, surface exclusion. The four tra genes identified in Tra1 are shown as traA, B, C and D in order shown on the map. The major part of Tra1 may be involved in pilus formation. traE is located in Tra2. The direction of the plasmid transfer is shown by the arrow.

group plasmid and has been shown to be identical to RP4 physically by restriction analysis. Genetic analysis of its tra region in Pseudomonas aeruginosa has revealed at least seven complementation groups (Chandler and Krishnapallai, 1974; Stokes et al., 1981). Genetic analysis of the representative mutants from different complementation of R18 and RP4 may shed light on the wide host range nature of these plasmids.

The W transfer system.

The IncW group of plasmids like the IncP and IncN plasmids are also considered to have a wide host range since they can be transferred and maintained by bacterial species outside the Enterobacteriaceae. Currently there are only three plasmids that belongs to this group. They are S-a, R7K and R388 (Bukhari et al., 1977). These plasmids, like the P and N group plasmids, transfer more efficiently when conjugation is carried out on solid media surfaces than in liquid media (Bardley, 1980). They do not specify any fertility inhibition system, although in the presence of an IncP group plasmid in the same cell, the transfer of W group plasmid is reduced by a factor of 10^{-6} indicating some interaction with the P-tra system (Yasoff and Stanisich, 1984). In addition, a small inhibitory effect of a W group plasmid on the transfer of an IncP group plasmid has also been observed (Olsen and Shipley, 1975). A further manifestation of a probable

interaction between the IncP, IncW and IncN plasmids is the existence of phages whose host range includes bacteria carrying any one these three groups of plasmids (Olsen et al., 1974; Coetzee et al., 1979). A double-stranded DNA phage, bacteriophage K7, that specifically infects the cells carrying an IncW group plasmid has been isolated (Morris et al., 1980). Cells carrying IncW plasmids also have the ability to kill K. pneumoniae cells used as recipients during conjugation like IncP plasmids (20%) but to a lesser extent than killing by IncN plasmids (90%) (M. Rodriguez, M.Sc. thesis, Carleton University, 1981).

All three members of the IncW group specify the production of morphologically and serologically similar pili, the W pili (Bradley, 1978). One structural feature of W pili is their pointed tips. Phage PRD1 was found to be attached to the tips of these pili and to the cell surface. It has been suggested that the transfer of W plasmids is inhibited by the adsorption of PRD1 implicating the involvement of W pili (tips) in conjugation (Bradley, 1978). No RNA containing phage for this group of plasmids has been reported.

Very little information is currently available on the W transfer system. In R388, the transfer function has been assigned to a minimum of a 15 Kb region by the generation of in vitro deletion derivatives (Ward and Grinstead, 1978). Using deletion derivatives and molecular

cloning of fragments, two transfer regions totalling approximately 12 Kb have been identified on the plasmid S-a (Tait et al., 1982). Deletion of the smaller of the two regions (2.6 Kb in size) reduced the transfer efficiency by 40 to 60%. Deletion of the larger region (9.5 Kb in size) reduced the transfer efficiency to less than 2%. No explanation for such reduced but still detectable transfer was offered. When present in an oncogenic strain of Agrobacterium tumefaciens the larger tra region has been shown to inhibit oncogenesis. Figure 4 (page 57) shows the organization of the W group plasmid S-a.

Other tra systems.

Although a number of donor specific bacteriophages have been isolated which are specific for cells carrying plasmids of any one of the several incompatibility groups, no information is available on the tra systems specified by any of these groups. The presence of morphologically and serologically distinct pili for these groups (Bradley, 1980a) implies that novel system may exist for each of these groups. Preliminary reports have appeared on transfer mediated by IncHI and IncJ plasmid groups (Taylor, 1983; Nugent, 1981).

From this literature review which was concluded in Sept., 1984, it is apparent that our current understanding of bacterial conjugation is dominated by the study of F

Fig. 4. Map of the IncW group plasmid S-a. Cm^r , Chloramphenicol resistance; Su^r , Sulphonamide resistance; Sm^r , Streptomycin resistance; Gm^r , Gentamycin resistance; Sp^r , Spectinomycin resistance; Km^r , Kanamycin resistance; Tp^r , Trimethoprim resistance.

and F-like plasmids. However, the transfer systems of F may not be mimicked by other tra systems. This is suggested by the presence of more than one tra region in the P type and the W type of plasmids. The broad host range plasmids of the IncP, IncW, and IncN group appear to be related. A study of a member of each group may help understand not only the spread of antibiotic resistance by bacterial conjugation but also the evolution of these tra systems. As indicated earlier, this study and thesis concerns the N tra system

MATERIALS AND METHODS

Bacterial strains, plasmids and bacteriophages.

The plasmid-less bacterial strains used are listed in Table 1. The plasmids used or derived in this study are listed in Table 2. In addition, a large number of strains carrying transposon Tn5 insertions in pCU1 and pCU109 (see Table 2) were obtained as part of this study. They are listed with information pertaining to them in Appendix I. The bacteriophages IKE and PRD1 have been described (Khatoun *et al.*, 1972; Olsen *et al.*, 1974). IKE is from this laboratory and PRD1 from R.H. Olsen. The λ phage vector of the transposon Tn5 (λ b221 rex::Tn5 cI857 Oam29 Pam80; abbreviated hereafter as λ ::Tn5) was obtained from G. Walker and is from the collection of M. Fox (Shanabruch and Walker, 1980).

Media and Chemicals.

Penassay broth (Antibiotic medium No. 3, PB) of Difco laboratories with or without 1.5% agar (Difco-laboratories) was used as the standard organic medium to which antibiotics were added as required. In solid media (e.g. Penassay Agar, PA), the concentrations (ug/ml) of the various antibiotics used were: Ampicillin (Ap), 30; Chloramphenicol (Cm), 30; Kanamycin (Km), 50; Nalidixic acid (Nal), 50; Rifampicin (Rif), 100; Streptomycin (Sm), 50; Spectinomycin (Sp), 50; and Tetracycline (Tc), 20. For liquid media, one-half of these concentrations were used.

^aThe strains listed are all plasmid-free. In addition to these strains, a large number of strains carrying pCU1 or its derivatives were constructed and used in this study. Such plasmid carrying strains are referred to in the text with the plasmid designation following the designation of the plasmid-free strain. For example strain C08/pCU1 is a derivative of C08 carrying pCU1. Plasmid carrying strains were constructed as described on page 63

^bArg-, His-, Met-, Pro-, Pur-, Thy-, Thi-, Leu-, Cys-, Thr-, dependence on arginine, histidine, methionine, proline, pyrimidine, purine, thymine, thiamine, leucine, cysteine, threonine; ara, gal, mtl, xyl arabinose, galactose, maltose, xylose utilization; RecA-, general recombination deficiency; Ap, Ampicillin; Rif, Rifampicin, Nal, Nalidixic acid; Str, streptomycin; r or s, resistance or sensitivity.

TABLE 1.

Bacterial strains^a

Strain	Relevant marker ^b	Source	Reference
<u>E. coli</u>			
C08	Arg- His- Lac- Met- Mtl- Pro- Pur- Pyr- Thy- Nal ^r Sm	K.B. Low	Bachmann (1972)
CR34	Thr- Leu- Thi- Thy-	F. Bonhoeffer	Bonhoeffer (1966)
WA802	Met- Gal- Lac-	R. Watson	Wood (1966)
C600	Thr- Leu- Thy- Thi-	S.N. Cohen	Bachmann (1972)
HE101	Pro- Leu- Thy- Thi- RecA-	Boyer	Bolivar et al. (1977)
JC7623	Thr- Leu- Thi- Lac- Gal- Ara- Xyl-	A. J. Clark	
GS500	same as HB101, Rif ^r	G. Selvaraj	This laboratory
CSH4	Trp- Lac- Str ^r Thi-	H. Yamazaki	J. Miller (1972)
KL16-99	RecA-	K.B. Low	Bachmann (1972)
<u>Klebsiella pneumoniae</u>			
M5a1	Prototroph, Ap ^r		McNeil et al. (1978)
<u>Salmonella typhimurium</u>			
LT2	Cys-	J. Brenchley	

^a Only those plasmids that have been constructed and used in more than one aspect of this study are in this table. In addition, a large number of Tn5 insertion derivatives of pCU1 and pCU109 are listed in Appendix I with properties associated with them.

^b All regions are Kb coordinates of pCU1 in the clockwise direction. Where the source or derivation is not indicated, the derivation is part of this study and is described in the text.

^c Abbreviations used: Ap, Ampicillin; Km, Kanamycin; Rif, Rifampicin; Tc, Tetracycline; Sm, Streptomycin; Sp, Spectinomycin; Tra, Conjugal transfer; IKE and PRD1, bacteriophages IKE and PRD1, Kil, killing of Klebsiella pneumoniae; + or -, presence or absence of a phenotype; r or s, resistance or sensitivity..

TABLE 2.
Plasmids derived and used¹

Plasmid	Region of pCU1 ⁰ (if present)	Source of derivation	Phenotype
pCU1		Konarska-Kozlowska and Iyer, 1981b.	Tra ⁺ Ike ^S PRD1 ^S Kil ⁺ Ap ^r Sp ^r Sm ^r
pCU10	5.1-33.2	---	Tra ⁻ Ike ^r PRD1 ^r Kil ⁻ Ap ^r Sp ^r Sm ^r
pCU11	0-28.8	---	same as pCU10
pCU16	27.5-25.3	---	same as pCU10
pCU20	0-27.2	---	same as pCU10
pCU102	16.5-2.8		Tra ⁻ Ike ^S PRD1 ^S Kil ⁻ Ap ^r Sp ^r Sm ^r
pCU53	27.3-3.8		Tra ⁻ Ike ^S PRD1 ^S Cm ^r
pCU54	21-26		Ap ^r Sm ^r Sp ^r Cm ^r
pCU55	11.5-15.7		Cm ^r
pCU56	7-11.5		Tra ⁻ Mob ⁺ Cm ^r
pCU57	0-8.2		Tra ⁻ Ike ^S PRD1 ^S Mob ⁺ Cm ^r
pCU58	27.5-34 and 5.1-8.2		same as pCU57
pCU405	27.5-34 and 5.1-11.2		Tra ⁻ Ike ^r PRD1 ^r Kil ⁻ Cm ^r Mob ⁺
pCU404	7-11.5		Tra ⁻ Mob ⁺ Ap ^r Km ^r
pCU406	3.8-7.		Ap ^r Km ^r
pCU411	27.3-3.8		Tra ⁻ Ike ^S PRD1 ^S Kil ⁻ Ap ^r Km ^r
pCU412	21-26		Ap ^r Sp ^r Sm ^r Km ^r
pCU29	pCU1::Tn5		Tra ⁺ Ike ^S PRD1 ^S Kil ⁺ Ap ^r Sp ^r Sm ^r Km ^r
pCU101	27.5-3.2		Tra ⁺ Ike ^S PRD1 ^S Kil ⁺ Cm ^r
pCU51	8.2-21		Km ^r
pCU231	pCU1::Tn5		same as pCU29
pCU105	27.5-10.2		same as pCU101
pCU66	pCU1::Tn5		same as pCU29
pCU109	27.5-11.2		Tra ⁺ Ike ^S PRD1 ^S Kil ⁺ Cm ^r
pCU167	pCU1::Tn5		Tra ⁺ Ike ^S PRD1 ^r Kil ⁺ Ap ^r Sp ^r Sm ^r Km ^r
pCU184	pCU1::Tn5		same as pCU167
pCU66	pCU1::Tn5		same as pCU167
pCU401	31.4-21		Tra ⁺ Ike ^S PRD1 ^r Kil ⁺ Km ^r
pCU402	31.3-21		same as pCU401
pCU403	31.6-21		same as pCU401
pACYC184		Chang and Cohen, 1978	Cm ^r Tc ^r p15A
pMK2004		Kahn <i>et al.</i> 1979	Ap ^r Km ^r Tc ^r ColEI
RP4		Bukhari <i>et al.</i> 1977	Ap ^r Km ^r Tc ^r P-group

Luria-broth (LB) consisted of 1.0% tryptone, 0.5% yeast extract, and 0.05% NaCl with the pH adjusted to 7.0 with 1N NaOH prior to sterilization. R-top agar consisted of 1% tryptone, 0.1% yeast extract, 0.8% NaCl and 0.8% agar to which CaCl_2 and glucose were added after sterilization to a final concentration of 2 mM and 0.4% respectively. λ broth was 1% tryptone and 0.025% NaCl. λ YM was λ -broth with 0.01% yeast extract and 0.2% maltose. λ -bottom and λ -top agar had 1.2% and 0.7% agar respectively. Dilutions of λ ::Tn5 were made in λ dilution buffer (λ dil) (10mM Tris-HCl, pH 7.5 and 10mM MgSO_4). When growth on a defined medium was required, the minimal agar medium of Davis and Mingioli (1950) was used. The filter sterilized nutritional supplements were added to this medium at the following final concentrations (ug per ml): thiamine, 1; thymine, 2; L-amino acids, 40 and glucose, 0.2% (w/v). The restriction endonucleases were purchased from Boehringer-Mannheim, Montreal and New England Biolabs, Massachusetts. T4-DNA ligase and T4-RNA ligase were purchased from New England Biolabs. Antibiotics were from Bristol-Myers, Montreal or Sigma Chemical Co.

Construction of strains carrying plasmids.

Conjugative plasmids were introduced into strains by a spot-conjugation method (see Determination of plasmid associated phenotypes) and non-conjugative plasmids by

DNA-mediated genetic transformation (see Genetic Transformation, p73).

Test for recA mutation.

The presence of a recA mutation in E. coli, HB101, was identified by the hypersensitivity of the strain to ultraviolet radiation. An overnight culture of HB101 was diluted 100-fold and allowed to grow for 2 h at 37° C. A sample of the culture was applied as a strip on 1/3 of a PA plate with a sterile cotton swab. As controls, C600 (a known RecA⁺ E. coli strain; Bachman, 1972) and K12-99 (a RecA⁻ mutant; Low, 1968) were spread on one-third each of the remaining portion of the same PA plate. The UV source was a mineralight-UV5-11 lamp (254 nm) at a distance of 15 cm (2 ergs/sec/mm²). The plate was covered prior to exposure. One tenth of each strip was exposed at a time with an increasing interval of 5 sec each, leaving the rest unexposed. The plate was incubated at 37° C in the dark. The growth of the test strain was then compared to the RecA⁺ and RecA⁻ controls.

Determination of the frequency of transfer.

The conjugation protocol which was routinely used was based on the observations of Dennison and Baumberg (1975) that N-group plasmids transfer efficiently when matings are carried out on the surface of solid media. 0.5 ml of an exponentially growing plasmid donor culture

in PB was mixed with 4.5 ml of a similar overnight culture of the recipient. The mixture was filtered through cellulose nitrate filters (0.45 μ m porosity, 45 mm in diameter). The filter was transferred to a prewarmed PA plate with the cell surface away from the agar surface and the plate was incubated at 37°C for 1 h. At the end of the incubation period, the filter was placed and the cells were suspended in 5 ml of saline (0.85% w/v). Serial dilutions were plated on selective media and the number of transconjugant colonies were counted after 24-48 h of incubation at 37°C. Donor and recipients plated separately on selective media served as controls. After two purifications on selective media, transconjugants were analysed further for their plasmid-associated phenotypes.

Determination of the frequency of killing of the *Klebsiella pneumoniae*.

A quantitative procedure for the detection of killing was used as described by M. Rodriguez (M. Sc. Thesis, Carleton University, 1981). A donor to recipient ratio of 20:1 was used. Mating was at 37°C for 1 h as described for conjugation. The survivors were scored on minimal agar. Matings with *E. coli* strains with and without pCUI were used as positive and negative controls.

Determination of plasmid associated phenotypes.

To determine sensitivity or resistance to

antibiotics, cells from a single colony were transferred with sterile toothpicks to PA plates containing antibiotics and incubated at 37°C. To determine whether a strain was transfer proficient in conjugation (Tra⁺), the following procedure was used. An overnight culture of the strain in PB was diluted 1:20 in PB and allowed to grow for 2.5 h at 37°C. After this period, 0.05 ml of the culture was spotted on a PA plate containing rifampicin (Rif) and any one of the appropriate antibiotics, the surface of which had been previously spread with 10⁸ cells of the Rif-resistant GS500. Selective plates that were not spread with the recipient strain or spread and spotted with the pCU1 parental strain were used as controls.

Another spot mating test that was used for screening a large number of colonies for conjugal transfer has been described by M. Rodriguez (1981). Cells from a single colony were transferred by a sterile toothpick to one of 25 individual compartments of a sterile plastic culture container (ELISA, Milano, Italy) each containing 0.7 ml of LB. The container was incubated for 12-15 h at 37°C. Using a sterile 25 multipoint inoculator, drops of all donor strains were spotted simultaneously, first onto a plate containing one of the antibiotics to confirm the presence of a plasmid marker and then to different selective plates where 0.2 ml of the recipient had been previously spread. Drops of donors spotted onto the

selective media without recipient served as the controls. If killing of Klebsiella pneumoniae was to be tested simultaneously, then the last selective plate was minimal glucose media spotted and spread with 0.2 ml of 10^8 cfu/ml of K. pneumoniae.

Sensitivity of strains to phages IKE and PRD1 was determined by a spot test (Konarska-Kozłowska and Iyer, 1981b). 0.1 ml of an overnight culture to be tested was spread on a PA plate and dried. 0.05 ml of a high titre of phage suspension (10^{11} pfu/ml) was spotted and air-dried. An area of clearance at the spot was observed in those instances where the culture was phage-sensitive.

Procedures involving the use of bacteriophages.

Phage lysate of IKE and PRD1.

High titres of phages were prepared as follows. 0.1 ml of phage dilutions were mixed with 0.2 ml of an E. coli strain carrying pCU1 for IKE and Salmonella typhimurium LT2 carrying RP1 for PRD1; 2.5 ml of molten R-top agar was mixed and poured on PA and incubated at 37°C for 15-18 h. Using a sterile glass slide, the R-top agar from plates that showed confluent lysis was collected in a sterile tube. It was vortexed thoroughly and then spun down at 27,000 Xg for 25 min. The supernate was transferred to a sterile tube. This procedure yielded 10^{10} - 10^{11} plaque forming units per ml.

Preparation of λ ::Tn5 lysate.

An overnight culture of C600 grown in λ YM was used for the propagation of λ ::Tn5. The procedure used for obtaining high titre phage was similar to that used for IKE and PRD1 with the following modifications. λ -top and λ -bottom agar were used in place of R-top and R-bottom agar. C600 and λ ::Tn5 were incubated at 37°C for 20 min prior to overlay. Chloroform (0.5 ml) was used to effect a complete lysis of cells before harvesting the phage. A few drops of chloroform were added to the λ ::Tn5 phage suspension prior to storage at 4°C. The phage lysate was assayed by the plate dilution technique as described by Miller (1972).

Plasmid DNA manipulations.

Isolation of plasmid DNA.

Three procedures were used for the isolation of plasmid DNA.

Boiling procedure. The "boiling method" as described by Holmes and Quigley (1981) was used for the isolation of plasmids of a size of 15 Kb or less. 5 ml of an overnight culture in LB with selection for the maintenance of plasmid was spun down in a Sorvall table-top centrifuge at 10,000 Xg for 10 min. The pellet was resuspended in 0.35 ml of STET buffer, 8% (w/w) sucrose, 0.5% (v/v) Triton X-100, 50mM EDTA (pH 8.0), 10mM Tris-HCl (pH 8.0) and

transferred to 1.5 ml Eppendorf tube. 25 ul of freshly prepared lysozyme (10 mg/ml in 10mM Tris-HCl, pH 8.0) was added and mixed by vortexing. It was placed in a boiling water bath for 40 seconds and centrifuged for 10 min at 14,000 Xg in an Eppendorf centrifuge (model 5412). The flocculent material was removed with a sterile wooden toothpick. 0.35 ml of cold isopropanol was added and the tube was held at -20°C for 1 h. The tube was centrifuged at 14,000 Xg for 15 min. The pellet was dried in vacuum and dissolved in 40 ul of distilled water and used without further storage. This procedure yielded enough plasmid DNA for two restriction endonuclease digestions.

Rapid alkaline extraction procedure. This plasmid DNA preparation method useful for small volumes of culture was as described by Birnboim and Doly (1979) with slight modifications. An overnight culture from a single colony in 2.5 ml of selective PB was grown on a roller drum. 1.5 ml of this culture was transferred to Eppendorf tubes and cells were pelleted at 14,000 Xg for 30 sec to 1 min. Cells were resuspended in 100 ul of solution I (10mM EDTA, 25mM Tris pH 8.0; prepared fresh prior to use from stock solutions of 0.5M EDTA, 2M Tris pH 8.0) by vortexing. It was incubated on ice for at least 15 min. Then 200 ul of solution II (0.5 ml 25% SDS, 0.67 ml 3M NaOH and 8.8 ml dH₂O prepared fresh prior to use) was added and mixed by inversion. At this stage the solution became viscous and

clear. After 5 min of incubation on ice, 150 ul of solution III (3M Sodium Acetate, pH 4.8) was added. It was kept on ice for a further period of 30 to 60 min. the white precipitate that was formed was spun down at 14,000 Xg for 15 min. Since the white precipitate tends to redissolve, only 4-6 tubes were spun down at a time making certain that the white precipitate is pelleted to leave a clear supernate. 400 ul of the clear supernate was transferred to a new 1.5 ml Eppendorf tube.. 1 ml of cold ethanol was added and the contents were mixed by inversion. The tubes were then transferred to a dry ice-ethanol bath for 15-20 min. After centrifugation at 14,000 Xg for 5 min, the alcohol was drained off. The pellet was dried in a vacuum dessicator to remove traces of alcohol and then dissolved in 100 ul of solution IV (100mM Sodium Acetate, 50mM Tris, pH 8.0) by vortexing gently. After adding 0.25 ml of absolute ethanol, the tubes were transferred to a dry ice-ethanol bath for 10 min. The precipitate was dried in a vacuum dessicator to complete dryness and dissolved in 50 ul of sterile dH₂O. 15 ul of this preparation was enough for restriction endonuclease digestion or transformation.

Clear lysate procedure. This procedure was as described by Clewell and Helinski (1969) with the modification described by Humphrey et al. (1975).

An 800 ml culture of a strain carrying a plasmid

was grown in PB with appropriate antibiotic(s) to late logarithmic phase. The cells were pelleted at 10,000 Xg for 10 min at 4°C in a Sorvall GSA rotor. The pellet was resuspended in a total volume of 30 ml of 25% sucrose in 0.05M Tris, pH 8.0. 5 ml of a freshly prepared lysozyme solution (0.1 gm of 3X crystallized lysozyme from Sigma in 10 ml of 0.25M Tris-HCl pH 8.0) was mixed by swirling the mixture. It was held on ice for 10 min. 15 ml of 0.25M EDTA, pH 8.0 was added and the mixture was held on ice for another 10 min with occasional swirling. The lysis was completed by the addition of 5 to 10 ml of a detergent mixture (1% Brij-58, 0.4% Sodium deoxycholate, 0.0625M EDTA and 0.05M Tris pH 8.0). With most of the E. coli strains, lysis was achieved in 1-5 min as indicated by an overall increase in viscosity. The chromosome-membrane complex was pelleted out of the suspension by centrifuging at 35,000 Xg for 30-40 min in a Sorvall SS34 rotor. To the supernate recovered in this step, NaCl was added to a final concentration of 0.5M. This was followed by the addition of PEG 8000 to a final concentration of 10% (w/v). The mixture was swirled gently and left at 4°C overnight. The following day, the PEG precipitate was pelleted in a Sorvall GSA rotor at 2,000 Xg for 5 min. The pellet was resuspended in 4 or 7 ml of TE buffer (0.01M Tris-HCl, 0.001M EDTA, pH 8.0). 0.5 or 1 ml of 10 mg/ml stock solution of ethidium bromide (Et-Br) was added

and then enough CsCl was added to achieve a refractive index of 1.3885 or 1.3890 using a Bausch and Lomb refractometer. The mixture was held at 4°C for at least an hour prior to the removal of the precipitate by centrifugation at 10,000 Xg for 15 min. The sample was transferred to a nitrocellulose tube which was centrifuged at 18°C in a Beckman Ti75 or Ti50 rotor at 270,000 Xg for 40 h or in a VTi80 rotor at 300,000 Xg for 4 h. At the end of the period the tube was held under a long-wave ultraviolet lamp and the top of two bands which contained chromosomal DNA, open circular and linear plasmid DNA was removed with a pasteur pipette or syringe. The lower band which contained covalently closed circular (CCC) DNA was removed by puncturing the side of the tube with a 21 gauge needle, withdrawing the band and transferring it to a 15 ml Corex tube. Et-Br was removed by several successive washes of the sample with CsCl-saturated isopropanol until the sample was colourless. The DNA was precipitated with 2X volume of cold absolute ethanol and collected. TE buffer was added dropwise to dissolve the CsCl precipitate. This alcohol-precipitated plasmid DNA sample was left at -20°C for at least 2 h. The DNA was pelleted at 11,000 Xg for 15 min in a Sorvall SS34 rotor. The alcohol was poured off and the pellet was dried in vacuum, then dissolved in 0.2 ml of TE buffer. The alcohol precipitation step was repeated once.

Genetic transformation.

The transformation procedure was as described by Cohen et al. (1972). An overnight culture of E. coli strain was diluted 100-fold in LB and was regrown to an optical density of 0.85 at 590 nm (For most strains, this was found to correspond to approximately 2×10^8 cfu/ml). When a strain carrying an antibiotic-resistance plasmid was used as the recipient for genetic transformation, the antibiotic (resistance to which was determined by the plasmid) was added to the medium. This ensured the continued maintenance of the plasmid in the recipient. The cells were thoroughly chilled on ice for 10 to 20 min. All the solutions were chilled to 4°C prior to use. The rest of the procedure was carried out at 0 to 4°C. The cells were pelleted at 12,000 Xg for 10 min. The pellet was washed once with 0.5 volume of 10mM NaCl. For the strains HB101 and GS500 this wash was replaced by 30mM CaCl₂ wash since some lysis of these strains was seen when NaCl was used. The cells were pelleted again and the pellet was resuspended in 0.5 of the original volume of 30mM CaCl₂ and incubated on ice for 20-30 min. The cells were pelleted again and resuspended in 1/20th of the original volume of 30mM CaCl₂. 0.2 ml of these "competent" cells were used for each transformation reaction. 1-50 ul (0.2-2.0 ug) of DNA was added and the incubation was continued for 60 min on ice. In some

2

1.0

1.1

1.25

1.4

1.6

1.8

2.0

2.2

2.5

2.8

3.2

3.6

4.0

4.5

5.0

5.6

6.3

7.1

8.0

9.0

10.0

11.2

12.5

14.3

16.0

18.0

20.0

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experiments where a volume of plasmid DNA of more than 50 μ l was used, the final CaCl_2 concentration was adjusted to 30mM. At the end of the period of incubation, the mixture was "heat-pulsed" at 42°C for 2 min or at 37°C for 5 min, after which it was chilled for 5 min and diluted 5 to 10 fold with LB. After 45 min at 37°C, with shaking to allow time for the expression of a transformed property, the cells were plated on selective media. Transformant colonies were scored after 24-48 h incubation at 37°C. They were then screened for plasmid associated phenotypes.

Restriction endonuclease digestion and electrophoresis of DNA fragments.

Plasmid DNA preparations were digested to completion with several restriction endonucleases used separately or in combinations of two. The reaction was carried out in 30 μ l volumes with DNA digested to completion with appropriate restriction endonucleases in buffers recommended by the respective suppliers. When the DNA sample was to be digested with two enzymes, it was first digested to completion with the enzyme that required the lower salt concentration of the two for 3 h. The DNA was ethanol precipitated and spun down in a Beckman Microfuge centrifuge (Model 152) at 10,000 $\times g$ for 5 min, vacuum dried and then digested in an appropriate volume of buffer with the second enzyme. The reaction was terminated by heating to 70°C for 10 min or by th

addition of 5-8 μ l of the electrophoresis loading buffer (40% glycerol (v/v), 0.05% Bromophenol blue and 5mM EDTA, pH 8.0). Electrophoresis of DNA fragments was carried out in agarose slab gels (agarose concentrations varying from 0.7% to 1.2% (w/v) in different experiments) as described by Tanaka and Weisblum (1975). The gels were composed of agarose, 95 ml of distilled water and 5 ml of 20X E buffer (800mM Tris, 400mM Sodium acetate, 20mM EDTA, adjusted to pH 8.3 with glacial acetic acid). The agarose was dissolved by autoclaving, Et-Br was added to a final concentration of 0.4 μ g/ml and the gel cast. The dimensions of the gels were 140 X 120 X 3 mm.

The electrophoresis was carried out as described by Tanaka and Weisblum (1975) in 1X E buffer containing 0.4 μ g/ml of ethidium bromide. Before loading, the samples were mixed with 5-8 μ l of electrophoresis loading buffer. Vertical gel electrophoresis was carried out at 20 V for 14 h at room temperature or 75 V for 4 h at 10-20°C. HindIII-generated fragments of bacteriophage lambda DNA run simultaneously in one of the wells provided the molecular weight standards (Murray and Murray, 1975). The molecular sizes (Kb) of the fragments in increasing order of mobility were, 23, 9.3, 6.6, 4.5, 2.5, 2.2, and 0.5.

Gels were photographed under long-wave (approximately 360 nm) ultraviolet light with a polaroid

MP4 vertical land camera system using #25 red and #9 yellow filters attached to the lens.

Isolation of deletion mutants of a plasmid.

Deletions of plasmid DNA were obtained by two methods. In one, purified pCU1 DNA was treated with a selected restriction endonuclease XmaI and ligated with T4-DNA ligase (see Cloning of plasmid DNA fragments, p77), and the preparation was used to transform an *E. coli* strain by the transformation procedure described earlier (p73). In a second procedure, used with pCU1 or one of its derivatives, the ligation step was omitted. In both cases, an antibiotic resistance marker (Sp^r , Cm^r or Km^r) on the plasmid was used for the selection of transformants. Plasmid DNA was isolated from an adequate number of transformants and their restriction pattern examined by agarose gel electrophoresis after digestion with appropriate enzymes to confirm the deletion of a specific fragment(s).

Cloning of plasmid DNA fragments.

The vector DNA (pACYC184 or pMK2004) and the pCU1 DNA providing fragments to be cloned were separately digested to completion with the selected enzyme. The reaction was stopped by exposure to 70° C for 10 min. The digested DNA samples were mixed and precipitated with 2 volumes of cold ethanol. After 2 h at -80° C, the DNA was

spun down in a Microfuge for 4 min. The DNA was taken up in 50 ul of TE buffer. The precipitation step was repeated, and the DNA was then vacuum-dried and taken up in ligation buffer. Ligation was in 50 ul of buffer (50mM Tris-HCl pH 7.8, 10mM MgCl₂, 20mM dithiothreitol, 1mM ATP, 50 ug/ml of bovine serum albumin, 1-3 ug of DNA and 3 u/ug of DNA of T4-DNA ligase. The reaction was at 16°C for 16-18 h, after which the mixture was used for transformation. One of the vector plasmid-associated antibiotic resistance markers was used for selection. The recombinants were screened for "insertional inactivation" of expression of the other antibiotic resistance marker on the plasmid.

Selection of Tra⁺ recombinant clones.

The DNAs of a pCU1::Tn5 derivative and pACYC184 were digested to completion with HindIII. Ligation was performed as described (see Cloning of plasmid DNA fragments), and the DNA used for transformation (p73). After transformation the cells were diluted to 5 ml in LB and incubated for 2 h at 37°C to allow time for expression of the Tra phenotype. They were then used as donors in a conjugation experiment (filter mating, p64). Transconjugants were selected on plates containing Rif and Cm.

Isolation of transposon Tn5 insertion derivatives.

Transposon derivatives were obtained on plasmid pCU1 or its derivatives by the following methods.

An overnight culture of the E. coli strain carrying a plasmid to be mutagenized was grown in λ YM with appropriate antibiotic. It was then diluted 100-fold with similar fresh medium. It was regrown for 3.5 h and λ ::Tn5 phage particles were added. The multiplicity of infection (moi) varied from 0.1 to 10 in different experiments. The mixture was incubated at 37°C for 2.5 h. The cells were then pelleted, resuspended in λ -broth and plated on λ -bottom agar containing Km and Cm or Sp (plasmid specified markers). Cells without exposure to the phage and phage preparations alone served as the controls. The plates were incubated at 37°C for 48 h, and colonies that arose were purified and tested for the various plasmid specified phenotypes (see Determination of plasmid-specified phenotypes, p65).

The second procedure used was the modification of the above procedure. A total of ~5000 colonies that were obtained on the selective plates were pooled with 5 ml of saline and inoculated into 1 litre of PB with appropriate antibiotics and incubated at 37°C until maximum turbidity was attained. Plasmid DNA was extracted by the CsCl-EtBr density gradient centrifugation procedure (p70). The plasmid DNA preparation was used to transform competent

cells of an E. coli strain as described previously (p73). The colonies that arose were analysed for plasmid associated phenotypes.

Genetic complementation analysis

Tra⁻ pCU1::Tn5 derivatives were first introduced into HB101 (a RecA⁻ strain) by genetic transformation (p73). Competent cells of each one of these strains carrying one of the plasmids were transformed with plasmid DNA of a Tra⁻ pCU109::Tn5 derivatives. It should be noted that pCU1 and pCU109 are intracellularly compatible and can cohabit a bacterial cell. Transformants were selected on plates containing Sp and Cm and further screened for the presence of other resistance markers. An overnight culture carrying the two Tra⁻ plasmid derivatives and of each plasmid separately were grown in appropriate antibiotic containing media. Drops of each of these strains were placed on selective plates which was previously spread with GS500 as recipient. Selective plates without the recipient strain and spotted with the donor strain served as the controls. PA plates containing RifSp and RifCm were used for the selection of transfer of the pCU1::Tn5 and pCU109::Tn5 derivatives respectively. A patch of the growth on the selective plate after 24 h of incubation at 37°C was indicative of the transfer of the plasmid. Strains carrying two such Tra⁻ Ike^r PRD1^r derivatives of pCU1 and pCU109 were also tested for their

sensitivity to the phages IKE and PRD1.

For some complementation tests, it was necessary to transfer a region carrying a particular insertion from pCU109 to pCU1. This was accomplished by cutting the Tn5 insert with restriction enzyme PvuI or KpnI that cleaves derivative into 3 fragments and for which there is no site on Tn5 DNA. This linear DNA preparation was used to transform a recB recC sbcB strain, JC7623, carrying pCU1. Selection was for Km^r and a pCU1 marker (Sp^r). The transformants were screened for loss of Cm^r (pCU109 specified marker). They were further screened for the Tra phenotype and for sensitivity to phage PRD1. The expected position of Tn5 in pCU1 was confirmed by restriction analyses.

Transformation mediated transient heterozygosis technique.

Complementation tests on some of the Tra⁻ pCU109::Tn5 derivatives were done as follows. An HB101 strain carrying a Tra⁻ pCU109::Tn5 derivative was made competent with the CaCl₂ treatment as described (see Genetic transformation). The strain was transformed with 2-3 ug plasmid DNA from various Tra⁻ pCU109::Tn5 derivatives (with Tn5 mapping at different loci on the pCU109 physical map). After the heat pulse at 37°C for 5 min, the cells were chilled on ice for 5 min and then diluted to 2 ml with fresh EB. They were incubated at 37°C for 1 h with shaking to allow expression of the Tra

phenotype of the transformant. 1 ml of these cells was then transferred to two 1.5 ml Eppendorf tubes. 0.1 ml of a 10-fold concentrated late logarithmic culture of GS500 was added to one as a recipient. The cells were pelleted in the Microfuge for 60 sec and 1.0 ml of the supernate was discarded. The cells were resuspended in the remaining volume and then transferred to the surface of a cellulose nitrate filter (25 mm diameter, 0.45 μ m porosity) placed on a PA plate. Transformed cells without exposure to recipient and competent cells alone treated in a similar manner served as the controls. Mating was at 37°C for 2 h. The cells were resuspended in 2 ml of saline and dilutions were plated on plates containing Rif and Cm. Transconjugants colonies were counted after 24-48 h of incubation at 37°C.

9

RESULTS

From the review of the published literature, it is apparent that among the Enterobacteriaceae there are at least five known transfer (tra) systems and probably many more to be yet discovered. These tra systems are identified usually and most conveniently on the basis of sensitivity to bacteriophages that are specific to cells carrying a plasmid of any one group. Some of these systems, such as those mediated by the N, P and W incompatibility group plasmids, allow the plasmids to be transmitted to a wide range of Gram-negative bacteria (Datta and Hedges, 1972; Tardif and Grant, 1981). Tra systems mediated by these plasmids therefore deserve attention.

N group plasmids confer on their Escherichia coli host sensitivity to bacteriophage IKE (Khatoun et al., 1972) and PRD1 (Olsen et al., 1974), which are indicators of the presence of N pili, organelles that are probably needed for this system of conjugation to function (Bradley, 1979). Another common property of this group of plasmids is the mediation of the killing of certain strains of Klebsiella. It has been observed that when bacteria carrying a conjugative plasmid of this group are mated with a K. pneumoniae strain, a large fraction of the recipients is killed (Rodriguez and Iyer, 1981). This effect (the Kil phenotype) correlated with the transfer proficiency of the plasmid, raising the possibility that

the killing ability is specified by a gene or genes contained within the tra region.

The bacterial plasmid pCU1 is a self-transmissible prototype plasmid of the conjugative and incompatibility group N (Konarska-Kozłowska, 1981a, b). It is one of the three members of this group that are being studied currently, the others being R46 (Brown and Willetts, 1981) or its derivative, pKM101 (Langer and Walker, 1981; Langer et al., 1981), and N3 (Ando and Arai, 1981). The origin of pCU1 has been described (Konarska-Kozłowska and Iyer, 1981a, Konarska-Kozłowska and Iyer, 1980) and is the subject of detailed physical analysis in this laboratory. In addition to conferring the phenotypes characteristic of plasmids of the N incompatibility and conjugative group (Dennison and Baumburg, 1975; Khatoon et al., 1972), the plasmid also confers resistance to the antibiotics ampicillin, streptomycin and spectinomycin (Ap^r , Sm^r , Sp^r). The purpose of this study was to investigate those regions on the plasmid DNA that specified the N-tra system as a contribution to an understanding of this system and also the basis of its apparent relationship to sensitivity to certain phages and to the killing of Klebsiella pneumoniae. As a first step, the approximate location of the tra region on the pCU1 physical map was determined.

A) Approximate location of N-tra on the pCU1 physical map.

Konarska-Kozłowska (1981) has physically mapped

restriction sites on the DNA of pCUI using the following restriction endonucleases: EcoRI, BamHI, HindIII, PstI, PvuI, BglII, SmaI, BstEII, SalI, HpaI and Aval. The relative position of the restriction sites for some of these enzymes is shown in Fig. 5. pCUI is a 39 Kb plasmid. On the physical map, SalI¹ is arbitrarily chosen as the zero coordinate and all other restriction sites are placed in relation to this site. The superscript number refers to a particular site number (e.g. BglII⁵ refers to 5th BglII site from the zero coordinate in the clockwise direction). The number following a hyphen after the abbreviation for a restriction enzyme refers to the number of that fragment in increasing order of its mobility on an agarose gel; e.g. BglII-5 refers to the 5th largest fragment in size generated by restriction of a plasmid with BglII. In an effort to localize some of the pCUI specified functions, derivatives of pCUI were generated by the deletion of specific restriction fragments in vitro. pCUI DNA was digested to completion with different restriction endonucleases separately and such DNA preparations were used for transforming E. coli C600. The resulting transformants were selected for Ap^r or Sp^r and screened for the other plasmid associated phenotypes. Plasmid DNA from a sufficient number of transformants was

Fig. 5. A map of plasmid pCU1 showing some of the restriction sites relevant to this study. "?" signifies uncertainty concerning the clockwise terminus of tra. For a more detailed restriction map, see Fig. 23.

extracted and the deletion of specific fragment(s) was confirmed by restriction analysis of the derived plasmid. On the map shown in Fig. 5, the limit of the tra region in the anticlockwise direction was defined by the HindIII² site because

- a) deletion of the 2.1 Kb region between HindIII¹ and HindIII² had no effect on conjugation proficiency (derivative pCU16, Fig. 6; Konarska-Kozłowska, 1981) or tra-associated phenotypes (Table 2).
- b) deletion of the 12.1 Kb region between SalI² and SalI¹ (pCU20, Fig. 6) or the 10.2 Kb region between SalI⁴ and SalI¹ (pCU11, Fig. 6) completely abolished transfer and related functions (Table 2),
- c) deletion of the 10.2 Kb region between KpnI³ and KpnI¹ resulted in pCU10 which had a phenotype similar to pCU11 (Fig. 5).

These deletion derivatives thus allowed one to localize the N-tra region approximately between the 34 Kb - 0 Kb coordinates clockwise on the pCU1 map. How far the region exceeds in either direction could not be determined. However, it could not exceed beyond the HindIII² site in the anticlockwise direction. It also suggested that sensitivity to bacteriophages forms part of the tra region. Further analysis using in vitro construction of deletion derivatives in the clockwise direction as shown on this map was not possible because of

Fig. 6. Restriction maps of some of the plasmids constructed in this study. The positions of the restriction sites are indicated above the bar, which is a linear representation of the pCUI circular map. H, HindIII; Bg., BglII; Sa, SalI; Sm, SmaI; Bm, BamHI. The figures below the pCUI bar correspond to the coordinates shown in Fig. 5. The shaded portion indicates parts of the plasmid deleted. Insertion of Tn5 or pACYC184 is indicated by the additional bar and circle respectively. Plasmid phenotypes are indicated on the right.

PLASMID-SPECIFIED PHENOTYPE

	5a ^r 5a ^r 5c 5m B ₂ B ₂ Bg ^r Bg ^r 5m ^r H ₂ Bg ^r L	TRA	IKK	PRD	KIL
pCU1	3C 35 39 5 10 15 20 25	+	S	S	+
pCU16		+	S	S	+
pCU20		-	r	r	-
pCU102		-	S	S	-
pCU53		-	S	S	-
pCU10	pACYC184	-	r	r	-
pCU11		-	r	r	-
pCU29	Tn ^r	+	S	S	+
pCU101	pACYC184	+	S	S	-
pCU231	Tn ^r	+	S	S	+
pCU105	pACYC184	+	S	S	-
pCU68	Tn ^r	+	S	S	+
pCU109	pACYC184	+	S	S	+

the scarcity of the available restriction sites. It was inferred that the tra functions of the plasmid pCU1 were likely to be determined within the largest BglII fragment (BglII⁷ to BglII¹, Fig. 5).

B) Cloning of BglII fragments of pCU1.

BglII is the only restriction enzyme that has a fairly even distribution of sites on the pCU1 DNA. All the possible fragments of pCU1 derived upon complete digestion with BglII were cloned separately into the cloning vector pACYC184. The scheme used to clone the BglII fragments is given in Fig. 7. pACYC184 is a small multicopy, non-conjugative plasmid (4 Kb in size, approximately 20 copies/cell) with a pl5A replicon (Chang and Cohen, 1978). It specifies resistance to Cm and Tc. There are several unique restriction sites located within Cm and Tc that could be potentially used for the molecular cloning of the DNA fragments by the insertional inactivation of one of the resistance marker. The cloning of pCU1-BglII fragments was possible because pACYC184 has a single BamHI site and cleavage at this site gives rise to sequences that are largely complementary to those generated by BglII (5'-GATC-3'). However, the resulting recombinant molecules cannot be cleaved by either of the two enzymes. Thus, the cloned fragments cannot be recovered.

pCU1 DNA cleaved with BglII was mixed with pACYC184

Fig. 7. Scheme used for the molecular cloning of the BglII fragments of pCU1. The procedure is described in detail in MATERIALS AND METHODS.

ENDS GENERATED BY

DNA separately cleaved with BamHI (ratio of 2:1), the mixture was ligated and used to transform strain WA802 selecting for Cm^r transformants. Clones carrying one of the pACYC184 markers were then screened for the loss of the other marker, Tc. Insertion of a DNA fragment at the BamHI site inactivates the Tc^r gene (Chang and Cohen, 1978). Out of a total of 1,500 Cm^r colonies screened, 160 were found to be Cm^rTc^s (9.4% recombinants). These were further screened for the presence of the pCUI associated phenotypes and classified into the following groups (the number in parenthesis indicates the number of clones in that group): (I) Cm^r Ike^s PRD1^s (2); (II) Cm^r Ap^r Sp^r Sm^r (10); (III) Cm^r Tc^r (148).

Small scale plasmid DNA preparations were made by the alkaline extraction procedure from the Cm^r Tc^s clones to analyse the clones further. The approximate sizes of the plasmids were determined in comparison to the pACYC184 vector to allow identification of clones carrying different pCUI-BglII fragments. The presence of a particular fragment was determined by digestion with different enzymes. Since the BglII insert cannot be recovered, plasmid derivatives were digested first with HindIII and then with SalI. These two enzymes have sites that are flanking the BamHI site on pACYC184. Comparison to pCUI DNA digested with BglII allowed one to identify the particular BglII fragment that was cloned. Table 3

TABLE 3.

Recombinant clones derived from pCU1.

Plasmid	Size (Kb)	pCU1 fragment	Phenotype conferred					
			Tra	IKe	PRD1	Ap	Sp	Sm
<u>Cloning vector pACYC184</u>								
pCU53	19.2	BglIII-1	-	s	s	s	s	s
pCU54	9.0	BglIII-3	-	r	r	r	r	r
pCU55*	8.0	BglIII-4	-	r	r	r	r	r
pCU56	8.5	BglIII-5	-	r	r	s	s	s
pCU410	0.7	BglIII-7	-	r	r	s	s	s
<u>Cloning vector pMK2004</u>								
pCU411	19.6	BglIII-1	-	s	s	r**	s	s
pCU412	10.2	BglIII-3	-	r	r	r**	r	r
pCU404	9.7	BglIII-5	-	r	r	r**	s	s
pCU59	8.8	BglIII-6	-	r	r	r**	s	s

In addition, all recombinant derivatives of pACYC184 showed resistance to Cm and recombinants of pMK2004 showed resistance to Km; -, absence of a phenotype; +, presence of a phenotype, r, resistance; s, sensitivity.

*Although this plasmid did not show any pCU1 associated phenotype in *E. coli*, it showed immunity to killing in a *K. pneumoniae* strain by a strain carrying plasmid pCU1 (Gill, M. Sc. thesis, in preparation).

**Specified by pMK2004.

shows the fragments cloned and the phenotypes specified by them. These clones are described and discussed below.

i) pCU54. This is an Ap^r Sp^r Sm^r Cm^r Tc^S derivative. Its size and the phenotype it conferred were consistent with its being a recombinant of plasmid pACYC184 and BglII-3 (5 Kb) of pCU1. The presence of the fragment was confirmed by HindIII, EcoRI, SalI digestion. In vitro deletion derivatives generated with PstI have indicated that the Ap^r gene is located anticlockwise to PstI¹ (Fig. 5;

Konarska-Kozłowska et al., 1983). This allowed one to locate Ap^r to a 21 - 22 Kb (1 Kb) region on the pCU1 map.

ii) pCU55. This is a Cm^r Tc^S derivative with no known pCU1 associated phenotype in E. coli. Its size (8 Kb) indicated that the BglII³-BglII⁴ fragment had been cloned in it. Although no known phenotype can be associated with this fragment in E. coli, Gill (M. Sc. Thesis, Carleton University, in preparation) has shown that this fragment can confer upon K. pneumoniae, the property of immunity to killing by N-group plasmids, the Kim phenotype.

iii) pCU56. This is a Tra⁻ Ike^r PRD1^r Cm^r Tc^S derivative which carries the BglII²-BglII³ fragment of pCU1. Although it is Tra⁻, it is mobilized efficiently from the cells carrying the parental pCU1 (It is discussed further in Localization of mob, section J).

iv) pCU53. The plasmids from the two strains which showed the phenotypes Tra⁻ Ike^S PRD1^S Cm^r Tc^S were analysed by

restriction analysis. It was found that in both cases, the largest BglII fragment of pCU1 (BglII-1, 15.2 Kb) had been cloned. To determine the orientation of BglII-1 with respect to the vector DNA, they were digested with SalI. As expected the SalI-2 and SalI-3 fragments of pCU1 were unaffected. Fig. 8 shows the two possible orientations and the expected fragment patterns of the plasmids after SalI digestion. From the electrophoresis pattern of the two plasmids it was concluded that orientation I was the correct one in both plasmids. Thus these two plasmids were identical and one was designated as pCU53. The phenotypes conferred by this plasmid (i.e. IKe^S and $PRD1^S$) indicates that N-pili are produced by cells carrying this clone but that all the genes needed for transmission of the plasmid are not included in this clone. Bradley (personal communication) has confirmed this by electron microscopic observation of N pili.

None of the Cm^r Tc^S clones that were analysed for plasmid content (a total of 50) were found to carry BglII¹-BglII² and BglII⁴-BglII⁵ fragments of pCU1. It is now known that BglII⁴-BglII⁵ carries a region required for the stable replication and maintenance of pCU1 (Konarska-Kozłowska, 1981). A similar strategy was used to clone some of the BglII fragments of pCU1 on the vector pMK2004 (ColEI-type replicon; Kahn *et al.*, 1979), a plasmid compatible with pACYC184 (Chang and Cohen, 1978).

Fig. 8. Determining the orientation of the BglII-1 fragment of pCU1 with respect to pACYC184 in pCU53. pACYC184 has a single SalI site. The numbers of fragments expected were four. The possible orientation and the fragment pattern that would be generated from them is shown as Orientation I and Orientation II. The expected sizes of the fragments (Kb) in

Orientation I: 10.2, 4.7, 3.4, 1.0

Orientation II: 10.2, 7.2, 1.0, 0.6.

The 0.85% agarose gel electrophoresis of pCU53. The position of the smallest fragments that are not visible in the photographic reproduction but were visible on the gels and in the negatives are shown by the arrows.

SCHEME USED FOR DETERMINING ORIENTATION OF AN INSERT CARRYING BglII OF pCU IN CLONE

GEL PATTERN
OBTAINED WITH SalI

BglII INSERTION IS
IN ORIENTATION :

This vector codes for resistance to Ap, Km and Tc. Like pACYC184, it also has a BamHI site in the Tc region. pCUI digested with BglII and pMK2004 digested with BamHI were mixed, ligated and transformed into *E. coli* C600. A total of 900 Km^r colonies were screened for Km^r Tc^S Ap^r. 30 of them (~3.3%) were found to be Tc^S. Two of them showed Sp^r. It was therefore concluded that the BglII⁵-BglII⁶ was cloned. One of the Tc^S clones showed the Tra- Ike^S PRD1^S phenotype indicating that the BglII⁷-BglII¹ was cloned. Plasmid DNA isolated from some of them (total of 10) were screened for a clone carrying the BglII¹-BglII² fragment. None of them showed the presence of an 8.7 Kb (3.5 Kb BglII fragment + 5.2 Kb of pMK2004) plasmid. To clone this particular BglII-6 fragment of pCUI, a derivative pCUI01 was used (see section F). Digestion of pCUI01 with BglII generates two fragments, the larger one carrying Cm^r; the smaller one is the BglII¹-BglII² fragment. BglII fragments of pCUI01 were cloned at the BamHI site of pMK2004 and then the Km^r transformants were screened for the Tc^S Cm^S Ap^r phenotype. One of two such clones was used for restriction analysis. Although the cloned fragment could not be recovered (because of the way the clone was obtained) its size (8.7 Kb) indicated that it carried the BglII¹-BglII² fragment (Fig. 5).

These experiments allowed us to conclude that tra

of pCU1 extends beyond the BglII² site in the clockwise direction on the pCU1 map (Fig. 5). The limits of the region in the clockwise direction could not however be defined because of the scarcity of the available restriction sites in this region (see Fig. 5). To overcome this difficulty, transposon Tn5 was introduced into this and other regions of the plasmid so that the transposon DNA would provide new restriction sites at different plasmid locations.

C) Isolation of transposon insertions.

Transposons are continuous regions of DNA which can move from one replicon to another and which cannot exist as independent replicons. In addition to the above-stated purpose of providing artificial restriction sites, there are several distinct advantages in using transposons as mutagens.

- i) They not only inactivate the gene in which they are inserted but also the genes downstream from the site of insertion if the gene in which the transposon is inserted is a part of an operon.
- ii) They carry an easily selectable phenotype, such as resistance to antibiotics.
- iii) The position of the insertion can be mapped by restriction analysis.

We used the transposon Tn5, which is 5.7 Kb in size, specifies resistance to kanamycin, neomycin and

other amino-glycoside antibiotics, and is known to have restriction sites for HindIII within its boundaries (Jorgensen et al., 1979). Fig. 9 shows the sites for some of the restriction enzymes on the DNA of Tn5, fragments generated by them and the location of the Km^r.

C08/pCU1 was infected with $\lambda::\text{Tn5}$ ($\lambda\text{b221 rex}::\text{Tn5 cI857 Oam29 Pam80}$). This suicidal vehicle has the following properties. It is unable to integrate into the host chromosome because of the deletion of the att region; it has amber mutations in its O and P replication genes and therefore does not replicate in a su-host. Its use is described in MATERIALS AND METHODS. The Km^r colonies that arose upon mutagenesis were grown in a small volume and then mated with C600. It was reasoned that even though the insertion may inactivate the tra system soon after the insertion event, there would be sufficient amount of preformed Tra proteins in the mutant cells at the time of mating and that this would allow the plasmid carrying the insertion to be transferred. The majority of the Km^r colonies did not transfer the Km^r (Tn5 associated) marker but they did transfer Sp^r (pCU1 associated). Presumably, in these derivatives the transposon was inserted into the E. coli host chromosome. They were not analysed any further. A fraction of the colonies (0.8%) did transfer all plasmid markers and Km^r. Among these, we expected various classes including a Tra

Fig. 9. Partial restriction map of Tn5. The data is taken from Jorgensen et al. (1979). The dark lines indicate the inverted repeats (called IS50L and IS50R) of Tn5. Km^r, resistance to kanamycin. The fragments generated from digestion of Tn5 with a particular enzyme are given. The number refers to the size of a fragment in Kb.

class. Such transconjugant colonies were purified and checked for various pCUI associated phenotypes (see Fig. 10). The following classes of insertion derivatives were obtained.

i) $\text{Tra}^+ \text{IKe}^S \text{PRD1}^S \text{Kil}^+ \text{Ap}^r \text{Sm}^r \text{Sp}^r \text{Km}^r$. Except for Km^r , these are phenotypically indistinguishable from pCUI.

These were the potential candidates to localize precisely the ends of tra or to determine if the tra region forms a single contiguous segment.

ii) $\text{Tra}^+ \text{IKe}^S \text{PRD1}^S \text{Kil}^+ \text{Ap}^r \text{Km}^r$. pCUI is known to have four stem-loop structures which are characteristic of transposons in general (Konarska-Kozłowska *et al.*, 1983).

One of the regions of pCUI (the antibiotic region) is known to be unstable and gives rise to different

derivatives of pCUI which have lost either or both of the Sp^r , Sm^r markers (Konarska-Kozłowska, 1981). The class may have arisen by insertion of Tn5 into the Sp^r/Sm^r region. Since this region is both dispensable and not of primary interest to the present study, they were not analysed any further.

iii) $\text{Tra}^+ \text{IKe}^S \text{PRD1}^S \text{Kil}^+ \text{Sp}^r \text{Sm}^r \text{Ap}^S \text{Km}^r$. In these derivatives, the insertion of Tn5 inactivated the Ap^r gene. They were useful for the precise localization of this gene.

iv) $\text{Tra}^+ \text{IKe}^S \text{PRD1}^r \text{Kil}^- \text{Ap}^r \text{Sm}^r \text{Sp}^r \text{Km}^r$. The insertion in these derivatives totally abolished the Kil phenotype and

Fig. 10. The different classes of pCU1::Tn5 derivatives obtained after insertion mutagenesis. They were classified on the basis of Tra, sensitivity to bacteriophages IKE and PRD1, ability to kill Klebsiella pneumoniae and resistance to Ap, Sp, Sm. The numbers in parenthesis indicates the number of mutants in that class.

the strains were resistant to only phage PRD1. They could be useful in localizing any gene(s) involved in the killing of K. pneumoniae.

v) Tra⁻ Ike^r PRD1^r Kil⁻ Ap^r Sp^r Sm^r Km^r. These insertions would help define the region involved in production, assembly and function of N-pili.

vi) Tra⁻ Ike^s PRD1^s Kil⁻ Ap^r Sm^r Sp^r Km^r. Insertions in these derivatives may be expected to be involved in some aspect of conjugation other than N pilus formation presumably, conjugative DNA metabolism. Their existence also proves that the N-pilus alone is not sufficient for the Tra phenotype.

Tn5 insertions which were Ike^r but still transfer proficient were not obtained indicating that sensitivity to the N-specific phage Ike is tightly linked to tra.

D) Mapping Tn5 insertions on the pCU1 map.

In order to define the ends of tra as precisely as possible and to clone this entire region(s) insertion derivatives (class i) were isolated and mapped in the inferred 'end regions', i.e., in the HindIII²-SalI⁴ region anticlockwise and in the BglII²-BglII³ region clockwise (see Fig 5). The positions of all these insertions were mapped but are discussed here only for the insertion derivative pCU29, which proved to be particularly useful. The rationale used in mapping was as follows. Insertion of Tn5 in a particular restriction fragment is expected to

alter its mobility. If Tn5 has a site(s) for that particular enzyme, then the fragment in which insertion has occurred would be cleaved and new fragments would appear corresponding to the number of sites on Tn5. If Tn5 does not have a site for the enzyme, the insertion would result in a decreased mobility of the fragment in which it is inserted (the decrease corresponding to the size of Tn5).

The Tn5 insertion in pCU29 was mapped at 8.2 Kb on the pCU1 map, using HindIII, BamHI and SmaI. Tn5 has two HindIII sites and one site each for BamHI and SmaI (see Fig. 9). Cleavage of pCU29 DNA with HindIII and its comparison to pCU1 cleaved with HindIII shows three new fragments and the 37.5 Kb fragment of pCU1 is missing (Fig. 11A). The 2.1 Kb fragment of pCU1 is unaffected. The new 2.3 Kb fragment of pCU29 is the internal Tn5 fragment (Fig. 9). The 20.5 Kb and 18.7 Kb fragments are generated because of the cleavage of 37.5 Kb HindIII fragment of pCU1. Thus the possible locations are 17.3 Kb away from HindIII² in the clockwise direction at 6.0 Kb or 17.3 Kb away from HindIII¹ in anticlockwise direction at 8.0 Kb (see Fig. 5). To determine which of the two locations is correct, pCU29 digested with BamHI was compared with pCU1 digested with the same enzyme. The BamHI site is located asymmetrically on Tn5 and thus the possible locations based on the BamHI digest alone would

Fig. 11. Electrophoretic migration of restriction fragments.

A. Restriction patterns generated by various enzymes for pCU1 and pCU29, indicating the position of the Tn5 insertion in the latter.

The numerals by the side indicate the size of the fragments in Kb. In pCU29, the migration patterns of the following fragments are unaffected: BamHI, 6 Kb; SmaI 23 Kb; HindIII, 2.1 Kb. The 9.9 Kb fragment of the SmaI digested pCU29 is a doublet. Calculations of fragment sizes above 22 Kb are approximate.

B. HindIII restriction pattern of pCU101 compared to that of pCU29 and pACYC184.

A

B

be dependent on the orientation of Tn5 with respect to the pCU1 map. Assuming the BamHI site to be in the centre of Tn5 (the difference in the two BamHI fragments of Tn5 is 0.3 Kb which is within the mapping error) the position of Tn5 in pCU29 based on the BamHI pattern would be 12.2 Kb away from one of the BamHI sites of pCU1 (15 Kb pCU29-BamHI fragment - 2.8 Kb of Tn5-BamHI) i.e. at the 8.7 Kb or 0.1 Kb coordinates of pCU1. Thus only the position at 8.7 Kb matches with the position based on the HindIII data. This position was confirmed by digestion with SmaI (see Fig. 11A and Table 4). In this case, the 16.3 Kb fragment of pCU1 is cleaved by the insertion and two new fragments of identical size (9.9 Kb) which appear as a doublet in Fig. 11A are produced. The precise location at 8.2 Kb was based on the combined data for all the three enzymes and was further confirmed by the HpaI digest of pCU29. The orientation of the Tn5 insertion in pCU29 was determined by in vitro deletion of this plasmid using BamHI. The BamHI site in Tn5 is asymmetrically placed and changes in this site do not inactivate Km^r. pCU29 was digested with BamHI, and transformed into E. coli (selection for Km^r). Restriction analysis of the plasmid DNA from one such transformant showed that Km^r was clockwise to the BamHI site of Tn5. This derivative, designated as pCU51 is 15 Kb in size. Addition of the sizes of fragments generated with different enzymes from

TABLE 4.

Fragment sizes generated by the digestion of pCU1 and pCU29 with different restriction endonucleases^a.

Restriction endonuclease									
BamHI		SmaI		HindIII		HpaI		SalI	
pCU1	pCU29	pCU1	pCU29	pCU1	pCU29	pCU1	pCU29	pCU1	pCU29
33		23	23	37.5		8.3	8.3	27	
	23.1	16.3			20.5	7.2	7.2		21.8
	15		10.9(2X)		18.7	6.0	6.0		11.3
6.0	6.0				2.3*	5.5	5.5	10.2	10.2
				2.1	2.1		5.3*	1.1	1.1
						3.5	3.5	0.5	0.5
						2.6	2.6		
						2.0			
							1.8		
						1.6	1.6		
							0.6		
39	44.3	39.3	44.8	39.6	43.6	39.3	45	38.8	44.9

^aThe fragment sizes were calculated by the agarose gel electrophoresis of fragments with agarose concentrations varying from 0.85% to 1.2%. λ DNA digested with HindIII was used as standards (Murray and Murray, 1975).

2X represents that the fragments appeared as doublet.

* indicates that the particular fragment is generated from Tn5 (see Fig. 9).

pCU29 was consistent with the fact that only one copy of Tn5 was inserted (see Table 4 for comparison of pCU1 and pCU29). In membrane-filter matings, this derivative transmitted its antibiotic-resistance markers at frequencies only slightly lower than pCU1 (Table 5). Like pCU1 it also conferred sensitivity to phages IKE and PRD1 and showed a Kil^+ phenotype. Thus it was concluded that this particular Tn5 insertion had only a slight effect on conjugation proficiency and no detectable effect on related bacterial functions.

E) Localization of the ends of the tra region.

Using a similar strategy, a number of Tra^+ and Tra^- pCU1::Tn5 derivatives were mapped (see Fig. 12, p109 and Fig. 21 p142 for the position of Tra^+ and Tra^- insertions respectively; they are designated as pCU followed by a number). It should be noted here that none of the thirty-three Tra^+ pCU1::Tn5 derivatives that were used mapped between the coordinates 32 Kb - 8 Kb on the pCU1 map. The position of the left-end of tra as 31.5 Kb was defined by the three insertions pCU167, pCU184 and pCU66 on the pCU1 map. All three were transfer proficient. Fig. 12 shows that the Tra^+ insertions map at several distinct sites. In the clockwise direction, eight insertions between 8.2 - 9.8 Kb that were tightly clustered showed the Tra^+ phenotype and sensitivity to N pilus specific bacteriophages. However, the possibility of tra functions

TABLE 5.

Comparison of the conjugal transfer*
frequency of pCU1, pCU29 and pCU101.

Donor plasmid (in strain C600)	Marker selected	Transfer frequency	
		Average	Range
pCU1	Sp	22	8.5 to 64
pCU29	Sp	7.5	0.6 to 20
pCU101	Cm	33	4 to 66

*Mating was at a donor to recipient ratio of 1:10. Exponential phase donor and stationary phase recipient population were mixed on a 0.45 μ m sterile filter by suction; the filter was incubated with the cell surface away from the medium for 1 h at 37°C; the cells were washed off in saline, vortexed and dilutions plated on appropriate media for viable counts and for transconjugants; donors and recipients alone were used as controls. colonies were scored after 48 h at 37°C. Control experiments showed that at the dilutions used, secondary matings did not occur on the selective plates. The frequency of transconjugants (expressed as the number of transconjugants per 100 donor cells) varied considerably-- see figures above.

Fig. 12. Tra⁺ insertion derivatives of pCU1. The position of these insertions was mapped by procedures described in the text. The maximum mapping error is 0.5 Kb. For additional information related to these derivatives see Appendix I.

being determined by regions clockwise to the site of insertion in pCU298 cannot be ruled out solely on the basis of transposon mutagenesis. Three insertions between the coordinates 32.2 and 33.8 Kb (pCU171, pCU203, pCU176; for map positions of insertion in these derivatives see Fig. 21, p142) abolished both *Tra* and phage sensitivity. Insertions at 6.5 Kb and 7.8 Kb abolished transfer but not sensitivity to IKe and PRD1. This indicates that *Tra* and the related function of N-pilus-specific phage sensitivity is determined by a region on the plasmid that extends from 32.2 Kb to 7.8 Kb. The assigned positions of the insertions have an error limit of 0.5 Kb. Insertions at only one end of *tra* abolish the *Tra* phenotype without affecting phage sensitivity. All *Tra*-insertion derivatives were also K11-.

F) Cloning of a minimal region that includes *tra*.

The various *Tra*⁺ Tn5 insertion derivatives that were mapped between the coordinates 8.2 to 11.8 Kb provided HindIII sites which were used as sites for cloning. The scheme used is illustrated for pCU29 in Fig. 13. The cloning vector used was pACYC184 which has a unique HindIII site in its *Tc*^r gene. Insertion of DNA at this site abolishes the *Tc*^r phenotype (Chang and Cohen, 1978). The two plasmid DNAs were digested, ligated and transformed into C600 and after incubation to allow for the expression of *tra*, they were used as donors with GS500

cells as recipients as described in MATERIALS AND METHODS (page 73). It was reasoned that if tra is located entirely within the largest HindIII fragment of pCU29 (indicated by arrows in Fig. 13), any recombinant carrying this fragment would be self-transmissible thus allowing the direct selection of such a clone. Three colonies that appeared on the selective plates were purified. Plasmid DNA was reisolated from all the three recombinants, digested with HindIII and analysed by agarose gel electrophoresis to ensure that the desired fragment had been cloned (see Fig. 11B, p104). To determine if the pCU29-HindIII fragment was inserted in the two possible orientations with respect to vector pACYC184, all the recombinants were digested with SalI. It was found that in all three cases the DNA was inserted in the same orientation as shown in Fig. 13. The recombinant plasmid derived from pCU29 was designated pCU101 (Thatte and Iyer, 1983). Using a similar scheme, corresponding HindIII fragments were cloned separately from two other Tn5 insertion derivatives of pCU1, pCU231 and pCU88 (see Fig. 6). The recombinant plasmids derived from pCU231 and pCU88 were designated pCU105 and pCU109 respectively. Comparison of the HpaI digest of these plasmids indicate that their respective sizes were: pCU101, 24.5 Kb; pCU105, 25.5 Kb and pCU109, 27.5 Kb (see Fig. 14). BglII digest of these plasmids showed that they generate two BglII

Fig. 13. Scheme used to clone the region from pCU1 required for conjugal transfer into the HindIII site of pACYC184. The thick part of pCU29 is the region of transposon Tn5. The HindIII sites (arrowheads) define the 20.5 Kb fragment of pCU29 that was cloned into pACYC184. Similar procedures were used for the construction of pCU105 and pCU109. The Sp^r , Sm^r , Ap^r and maintenance regions are defined according to Konarska-Kozłowska *et al.* 1983. A detailed description of the procedure used for the selection of Tra^+ clones is given in MATERIALS AND METHODS. Tc* indicates that tetracycline resistance is not expressed.

Hind III DIGESTION
LIGATION TRANSFORMATION
of *E. coli* O-600

2 HOURS AT 37°C

E. coli HB101 rif^r

FILTER MATING

SELECT FOR Rif^r Cm^r

SCREENING FOR Tra⁺, lke^S, PRD1^S, Cm^r, Tc^r,
RESTRICTION PATTERN OF PLASMID DNA

Fig. 14.

A. 0.85% agarose gel electrophoresis of HpaI-digested DNA of pCU101, pCU105, and pCU109. The numerals by the side of the gel indicate the size of the fragment generated as calculated from the molecular weight standard HindIII digest of λ DNA (Murray and Murray, 1975). The arrow indicates the increases in fragment sizes in pCU101 to pCU109 due to pACYC184 (4 Kb) in these plasmids. The 0.5 Kb fragment in pCU109 is due to an additional HpaI site present in this plasmid in comparison to pCU101 or pCU105.

B. Gel electrophoretic migration pattern (in 1.2% agarose) of restriction fragments generated by the digestion of pCU105 and pCU109. Note that the orientations of HindIII-tra in these plasmids with respect to pACYC184 is opposite to one another.

fragments with BglII¹-BglII² of pCU1 intact and others showing a progressive increase in size from pCU101 to pCU109. As expected, pCU101 hybridized to 27.2 Kb to 10.6 Kb region of pCU1 (BglII⁷ to BglII¹; Konarska-Kozłowska and Iyer, 1983). It was thus concluded that all these insertions are in the BglII²-BglII³ region with pCU29 closer to BglII² and pCU88 to BglII³. The cloning data thus show an excellent correlation to the mapping position in these insertion derivatives by restriction analysis. The presence of an additional HpaI fragment (equivalent to the HpaI fragment located within BglII²-BglII³) in pCU109 in comparison to pCU101 and pCU105 indicates that the Tn5 insertion in the derivative from which it was derived (pCU88) is located between HpaI⁴ and BglII³ (Fig. 5). The orientation of HindIII-tra insert with respect to pACYC184 in pCU105 is similar to pCU101 but in pCU109 it is in the opposite orientation. In mating experiments (Table 5, shown only for pCU101), pCU101 and pCU105 were transmitted at a high frequency comparable to pCU1. They also conferred sensitivity to IKE and PRD1 but unlike pCU29 and pCU231, they did not confer the ability to kill the K. pneumoniae strain. It was therefore concluded that pCU101 and pCU105 carry an entire region necessary for the efficient conjugal transmission of these plasmids but that this region is insufficient for the killing of K. pneumoniae.

G) Localization of tra within the estimated 16 Kb tra region (between 31.5 and 36.2 Kb)

To determine if the entire 16 Kb region present in pCU101 is required for the Tra⁺ phenotype a total of 28 Tra⁻ insertion derivatives that were obtained by Tn5 mutagenesis (group v and vi) were mapped by procedures described in detail for pCU29. The position of all the mutants (total of 13) in group v was on the right side of tra of pCU1 as shown in the Fig. 21. Since they were Tra⁻ IKe^r PRD1^r, it was inferred that the pilus region is located between 31.5 and 36 Kb coordinates pCU1. This data confirms the results obtained with the in vitro deletion derivatives generated by SalI and KpnI. All the mutations in group vi (a total of 8) mapped within the 6 - 8 Kb coordinate region. As seen from Fig. 12 and Fig. 21 there is a 9 Kb region in which no insertion mutant mapped (Tra⁺ or Tra⁻). The method used to obtain these insertions thus did not allow us to saturate the tra region with transposon Tn5. One possible explanation is that a sufficient number of insertions have not been analysed and that when a large target molecule like pCU1 (39 Kb) was available (two and one-half times the tra region) insertions into regions outside the tra region prevent easy saturation of tra. The clustering of these insertions at the ends of the tra cannot be explained although it is possible that the "mating-out transposition

method" that was used, in the initial selection of Tra- mutants, may have favoured such insertions. It is also known that Tn5 transposition need not be completely random with respect to targets (Berg et al., 1983).

H) Attempts to saturate the central 9 Kb region of tra with transposon Tn5.

In an attempt to saturate the tra region we chose pCU109, since it has all the tra-related phenotypes specified by the parental plasmid pCU1 but is smaller. Another potential advantage would be to use these mutants against the pCU1::Tn5 mutants in genetic complementation tests (pCU1 and pCU109 are different and compatible replicons). CSH4/pCU109 was mutagenized with λ ::Tn5, and transductants were selected for Cm^r Km^r . Plasmid DNA was prepared from a pool of transductants. This DNA preparation was transformed into C600 and Km^r Cm^r transformants selected. It was expected that all the transformants would carry Tn5. A total of 1000 transformants were screened and among them approximately 70% showed the Tra- phenotype. The Tra- mutants were further analysed for sensitivity to bacteriophages IKE and PRD1. Based on these criteria the transformants fell into three groups: (a) Tra- IKE^S PRD1^S , (b) Tra- IKE^r PRD1^r and (c) Tra^+ IKE^S PRD1^S . Mutants that showed a Tra^+ Kil^- phenotype were not obtained. The plasmids in Tra- derivatives are designated as pVT (followed by a number)

and plasmids in Tra⁺ derivatives as pSV (followed by a number). A large majority of them fell into class (b) (237 out of 273 screened; 87%). Plasmid DNA from 52 Tra-Ike^r PRD1^r was isolated and analysed by agarose gel electrophoresis. In 15 of them deletions were detected upon restriction analysis. Two of them showed two insertions. These 17 derivatives were not used further. The remaining 35 insertions were mapped on pCU109 by a procedure similar to the one described for pCU29. Initially their approximate location on pCU109 was determined by restriction endonuclease digestion with KpnI and PvuI separately (Fig. 21; for detailed restriction map of pCU109 see Fig. 15, p118). There are two sites each for these enzymes on pCU109 DNA and none on Tn5 (Jorgensen et al., 1979). An increase in size of a particular fragment was considered to be due to an insertion in that fragment. Subsequently, the precise location of the insertion was determined using HpaI. HpaI cleaves the pCU109 DNA into 7 fragments. Fragments HpaI-4 and HpaI-5 are identical in size, and mapping the insertions in these two fragments was difficult (see Fig. 14). This problem was resolved by digestion with BglII. The positions of the insertions on pCU109 are shown in Fig. 15 and Fig. 17. The Tra- Ike^r PRD1^r insertions mapped within the region corresponding to the 32.5 to 2.5 Kb interval on the pCU1 map. The Tra- Ike^s PRD1^s insertions mapped within the

Fig. 15. Map position of the Tra^+ pCU109::Tn5 derivatives (designated as pSV__) on the physical map of pCU109. The thicker part of the figure is the vector pACYC184 and the thinner part is of the pCU88 HindIII-1. These insertions do not affect any of the phenotypes specified by pCU109.

region 5 to 8 Kb. There are two regions 1.3 Kb and 2.5 Kb in size in which none of the insertions mapped. Various possible reasons for this are considered in the

DISCUSSION. None of the Tra^+ insertions mapped within the 9 Kb central region. We do not know whether any particular Tn5 insertion actually disrupts a gene(s) required in conjugal transfer or whether it is merely polar on such a gene.

I) Localization of the region determining N pilus-specific phage sensitivity.

From the results based on the molecular cloning of the BglII-1 fragment of pCU1; it was concluded that the region on pCU1 determining sensitivity to IKE and PRD1 is a sub-sequence of the tra region extending from the coordinates 3.2 to 3.4 Kb. Since no insertions that abolished sensitivity to these phages were mapped near the right end of the tra region, we attempted to obtain a deletion mutant that would confer sensitivity to IKE and PRD1 without conferring the Tra^+ phenotype. SmaI cleaves pCU1 DNA at the 2.8 Kb coordinate (SmaI¹ site), approximately 1 Kb anticlockwise to the BglII¹ site (Fig. 5). Cleavage with SmaI produces blunt ends (Endow and Roberts, 1977) and E. coli cells are unable to ligate the blunt ends. E. coli cells transformed with SmaI digested pCU1 generated various derivatives but their size was smaller than expected (smallest derivative had a size of 9.5 Kb),

presumably because of the in vivo deletion generated (Konarska-Kozłowska, 1981). In order to avoid in vivo deletions, pCU1 was digested with XmaI (an iso-schizomer of SmaI which produces sticky ends), ligated and transformed into C600. The plasmid DNA from a resulting Sp^r transformant was then isolated and the deletion was confirmed by restriction analysis. pCU102, showed precise deletion of SmaI-2 and the extent of deletion in this plasmid is shown in Fig. 6 (p88). The strain carrying pCU102 was Tra- IKe^S PRD1 S indicating that the N-pilus region is included in a region from 32 - 2.8 Kb (Fig. 6). There are several Tn5 insertions mapped within the corresponding regions on pCU1 and pCU109 plasmids. pVT75 and pVT61 are the Tn5 carrying derivatives of pCU109 that are IKe^r PRD1 r (see Fig. 21 and Appendix I). These insertions map within 0.5 Kb of the SmaI 1 site indicating that the sequences required for sensitivity to phages end before SmaI 1 . Thus except for 1.3 Kb, the entire 9.8 Kb region is saturated with Tn5. There are insertions which are Tra $^+$ IKe^S PRD1 r (e.g. pCU167, group ii) confirming that sensitivity to PRD1 is not necessarily associated with the Tra $^+$ phenotype. Since the Tn5 insertions mapping within the pilus region are always Tra- IKe^r PRD1 r , it is possible that these mutations may define a region that encodes for a product which may modify the pilus for PRD1 sensitivity. To test this hypothesis, two deletion derivatives of

pCU101 were used. These deletions were generated by in vitro deletion of KpnI and SalI fragments of pCU101 (see Fig. 19) in a manner analogous to the deletions generated from pCU1. The deletion derivative generated by SalI digestion was designated as pCU57 and that from KpnI as pCU58. The deletion of specific fragments in pCU57 and pCU58 was confirmed by the restriction analysis. Both derivatives are PRD1^r due to deletion in the pilus region. These plasmid derivatives (p15A replicon) are compatible with pCU167 (pCU1 replicon). They were introduced into a strain carrying pCU167 by transformation. The deletion of SalI fragments to give rise to pCU57 results in the loss of a region 27.5 Kb to 0 Kb of pCU1. The strain carrying pCU167 and pCU57 did not show sensitivity to PRD1. The deletion of the KpnI fragments to give rise to pCU58 results in the loss of a region that corresponds to coordinates 35.9 to 5.1 Kb. A strain carrying pCU167 and pCU58 did show sensitivity to PRD1 indicating that the function determined is trans acting. The strain carrying pCU167 showed the production of pili that were morphologically similar to the N-pili indicating no gross alteration in the structure of the pilus (Bradley, personal communication). Taken together with the results obtained from the analysis of the Tn5 insertion derivatives, this leads to the conclusion that the plasmid region required for sensitivity to Ike and PRD1 extends

Fig. 16. Localization of the mob region of pCU1. pCU101 is a self-transmissible plasmid. pCU56 is a clone of the BglII²-BglII³ fragment of pCU1 onto pACYC184. It is Tra⁻ Mob⁺. pCU57 is a deletion derivative of pCU101. It is Tra⁻ Mob⁺. The only common region of these three derivatives is defined as the mob region. It is 1.2 Kb in size and is shown as a solid bar.

rightward from 30.6 at one end of tra to 2.8 Kb. This leads to an upper estimate of 11.2 Kb for the pilus region, which is a subsequence of tra. For sensitivity to PRD1 an additional region 1 Kb anticlockwise to the tra region is needed. This 1 Kb region is not required for conjugation.

J) Localization of a region required in cis for transfer (mob).

In the few tra systems that have been studied, transfer of the plasmid DNA during conjugation originates at a specific site known as the oriT region (origin of Transfer). There also exist some plasmids that are not self-transmissible but can be mobilized or donated with the help of a self-transmissible plasmid. There is evidence showing that the latter group of plasmids also have origins of transfer (Clark and Warren, 1979). oriT must, by definition, be present in cis on the plasmid being transferred. Thus, a region (called the mob region) that is required in cis on a plasmid for its transfer but which can be mobilized for transfer by gene and gene products provided by a separate plasmid in the same cell is likely to contain the oriT region. pCU101, a clone carrying a 19.4 Kb region (from 27.2 - 8.2 Kb coordinates) of pCU1 is self-transmissible and must therefore carry the mob region. The plasmid pCU53 carry BglIII-1 of pCU1 was not mobilized at a detectable level from a strain carrying

in addition, pCU1 (frequency of mobilization $<10^{-6}$). Thus pCU53 does not carry a functional mob region. However, a clone, pCU56 carrying BglII-5 (coordinates 7 - 11.5 Kb) was mobilized by pCU1 at frequencies comparable or even higher than the mobilizing plasmid (Table 6). The region common to pCU101 and pCU56 is 1.2 Kb in size thus indicating the position and size of mob (Fig. 16). To further confirm this location two deletion derivatives of pCU101, pCU57 and pCU58, were introduced into a strain carrying pCU1. These derivatives were mobilized as efficiently as the parental pCU1 supporting the location of mob between 7 - 8.2 Kb coordinates on the pCU1 map. Since there are several Tn5 insertions (on pCU1 and pCU109) that map within or close to this region, they were screened for cis-dominant transfer deficiency (see Genetic complementation analysis). None of the Tn5 insertion derivative showed this effect. To obtain such a derivative, a strain carrying pCU57 was mutagenized with $\lambda::Tn5$, plasmid DNA from a pool of transductants was transformed into HB101/pCU1 (host strain was chosen to be recombination-deficient (recA) to minimize transfer via homologous recombination of the two plasmids; pCU1 was used as mobilizing plasmid). The resultant transformants were then grown individually and checked for transfer of Cm^r and Km^r (pCU57 and Tn5 markers respectively) and Sp^r (pCU1 marker). In all of the 1575 colonies tested Cm^r

^aMatings were at a donor to recipient ratio of 1:10. Donors were grown in LB with selection for plasmid(s) markers. Exponentially growing donor and stationary phase recipient populations were mixed and collected on a 0.45 μ M sterile filter by suction; the filter was incubated on a prewarmed PA plate at 37°C with the cell surface away from the medium for exactly 30 sec at 37°C; the cells were washed off in saline immediately, vortexed and dilutions were plated on appropriate media or viable count and for transconjugants; donors and recipients alone were used as controls. Colonies were scored after 48 h at 37°C. The transconjugants colonies that arose on plates selective for the test plasmid were checked for the presence of the pCU1 marker. 12-15% of them showed Sp^r.

^bAll donor plasmids were in E. coli GS500.

^cCm was used for the selection of the test plasmid and Sp for pCU1

^dTransfer frequencies are calculated as number of transconjugants "per 100 donor cells". $<10^{-6}$ indicates that the transfer was not detected.

^eThe number represents the ratio:

$$\frac{\text{transfer frequency of the test plasmid}}{\text{transfer frequency of the pCU1 plasmid}}$$

from the same host background.

TABLE 6.

The frequency of mobilization^a of various pCU1 derivatives by pCU1.

Donor plasmid(s) ^b	Marker selected ^c	Transfer frequency ^d		
		pCU1	Test plasmid	Ratio ^e
pACYC184	Cm	-	$< 10^{-6}$	-
pACYC184/pCU1	Cm		$< 10^{-6}$	
	Sp	1×10^{-3}		10^{-3}
pCU57	Cm	$< 10^{-6}$		
pCU57/pCU1	Cm		4.5×10^{-3}	
	Sp	1×10^{-3}		4.5
pCU58	Cm		$< 10^{-6}$	
pCU58/pCU1	Cm		5×10^{-3}	
	Sp	2×10^{-3}		2.5
pCU56	Cm		$< 10^{-6}$	
pCU56/pCU1	Cm		5.5×10^{-3}	
	Sp	3×10^{-3}		1.8

could be transferred as efficiently as markers on the mobilizing plasmid, pCU1. To ensure that Tn5 was indeed present on the pCU57 plasmid, 35 transformants obtained from a transformation experiment in a plasmid-less host were mapped by restriction endonuclease analysis (Fig. 17). It should be noted that none of the insertions mapped in the 2.5 Kb region within the tra region in which previous attempts to obtain Tn5 insertions had failed.

K) Relationship of the tra region to the Kil phenotype.

It has been shown that E. coli cells carrying N group plasmids kill Klebsiella pneumoniae upon conjugation (the Kil phenotype). A previous study in this laboratory had shown that mutants could be obtained that separate these two functions (Rodriguez, 1981). However, the four mutants obtained in that study were generated by in vitro mutagenesis of the plasmid pCU1 by hydroxylamine treatment. Hydroxylamine causes a base substitution (point mutations) and thus the mutations in these mutants could not be conveniently mapped. In addition, two of them had undergone additional changes such as deletions. The Tra- Tn5 mutants discussed previously were also tested for the killing of K. pneumoniae. All of the large number of Tra- derivatives were also Kil-. Group (iv) mutants (pCU167, pCU184, pCU66) of pCU1 showed the Tra⁺ Kil- phenotype. The position of Tn5 in these derivatives was mapped by restriction analysis (Fig. 18). Two of three

Fig. 17. Position of Tn5 insertions on the physical map of pCU57. The plasmid is 13.0 Kb in size. The relevant restriction sites are shown on this map. These positions are mapped by procedures described in the text. Each derivative of pCU57 carrying a specific mapped Tn5 insert has been given a particular "pCU--" designation.

Fig. 18. Electrophoretic migration patterns (in 0.85% agarose) of restriction fragments generated from pCU167, pCU184, pCU66 and pCU1 with different enzymes.

a, pCU167; b, pCU184; c, pCU66; d, pCU1.

The numerals by the side of fragments indicates its size in Kb-as calculated using molecular weight standards from a HindIII digest of λ (Murray and Murray, 1975).

2X indicates that the fragments appear as a doublet

* indicates that the fragment is generated internally from transposon Tn5.

The differences in migration patterns of pCU167 or pCU184 and pCU66 are indicated by arrows. For HpaI fragment sizes of pCU1 see Table 4. The restriction patterns of pCU167 and pCU184 were similar with HindIII, SmaI, BamHI, PstI, HpaI.

BamHI

a a

A

HindIII

a a

B

SmaI

d a

C

BamHI

a b

D

HpaI

a b

E

mutants (pCU167 and pCU184) could not be distinguished on the basis of the gel pattern obtained with them after digestion with HindIII, BamHI and HpaI. They map at 31-32 Kb coordinates of the pCU1 map (Fig. 19). Table 7 shows the transfer and killing ability of the three in comparison to pCU1. They transfer at frequencies comparable to pCU1 but are unable to show killing at a detectable level. To determine the orientation of these Tn5 insertion on the the pCU1 map, the DNA from these plasmids was digested with BamHI and transformed into an E. coli strain. The Km^r colonies that arose were further screened for the loss of Sp^r, Sm^r and Ap^r. Plasmid DNA from such a strain was extracted and the deletion of specific fragments was confirmed by restriction analysis. The fact that Km^r deletion derivatives were obtained in this manner indicated that Km^r was clockwise to the BamHI site in these derivatives (see Fig. 19). The deletion derivatives obtained by such a procedure from pCU167, pCU184, pCU66 are designated as pCU401, pCU402 and pCU403 respectively. Fig. 19 indicates the extent of deletion in these derivatives. As expected, these deletion derivatives transferred Km^r but did not show the Kil⁺ phenotype. In addition to the loss of the Kil phenotype, these mutants were also PRD1^r (indicating the involvement of a plasmid region in both functions (see section I). To determine whether the Kil phenotype can also act in trans,

Fig. 19. Construction of plasmid derivatives to define the position on pCU1 of the two regions required for the killing of *K. pneumoniae*. The lines with circles at the end represent the Tn5 insertions. Their respective pCU designations are given by the side of circle. ----- indicates the part deleted and the number inside the triangle is the designation given to the resultant derivative.

Tra, conjugal transfer; mob, region required in cis for transfer. *kilA* and *kilB*, regions required in killing of *Klebsiella pneumoniae*; line with a filled circle, Tra-; line with a circle at the end, Tra⁺.

TABLE 7

Comparison of the efficiency of plasmid transfer^a and of killing of *K. pneumoniae*^b by *E. coli* strains carrying pCU167, pCU184, pCU66 and pCU1

Plasmid ^c	Marker ^d	Frequency of plasmid transfer (% of donors)	Fraction (%) of <i>K. pneumoniae</i> killed upon mating on filter
pCU1	Sp	20	70-90
pCU167	Km	7	0
pCU184	Km	8	0
pCU66	Km	1	0

^aTransfer of a plasmid was measured as described in MATERIALS AND METHODS at a donor to recipient ratio of 1:10. It is expressed as "per 100 donors" in 1 h mating at 37°C.

^bKilling was done at a donor to recipient ratio of 20:1. The survivors were scored on glucose minimal media, which effectively eliminated the auxotrophic donor.

^cPlasmids were maintained in *E. coli* C600.

^dMarkers used for the primary selection of transconjugants; in addition, all plasmids specify resistance to Ap and Sm and plasmids pCU167, pCU184 and pCU66 to Sp.

Gill (M. Sc. dissertation, in preparation; Carleton University) has tested strains carrying the plasmid pairs pCU167 - pCU57 and pCU167 - pCU58 for their ability to cause E. coli to kill K. pneumoniae. Complementation for Kil was seen with strains carrying pCU167 and pCU58 only (see section I). The above results indicated that transfer per se is not sufficient for the Kil phenotype and that a region between 30.8 Kb to 32 Kb is involved in the Kil and PRD1 sensitivity functions.

pCU101 (also pCU105) is a self-transmissible derivative of pCU1 that carries the above mentioned region. However it is unable to show the Kil⁺ phenotype indicating the need for an additional Kil region(s) present on pCU1 but not included in pCU101 (or pCU105; see Table 8). pCU109 which carries in addition to the region common to pCU101, a continuous 3 Kb region clockwise to mob (see Fig. 16) but separated by 1 Kb was both transfer and Kil proficient. Table 9 shows the killing efficiency by hosts carrying pCU1, pCU109 and pCU88 (from which pCU109 was derived). As can be seen from the Table the killing of K. pneumoniae by E. coli carrying either pCU88 or pCU109 is comparable to that shown by the strain carrying pCU1. We have assigned the kilA region at 30.8-32 Kb and the additional kil region on pCU109 as kilB. Since pCU167 carried the kilB region but had kilA inactivated (Fig. 20), this shows that kilB alone is not

TABLE 8

Comparison of the conjugal transfer frequencies^a and the efficiency of killing^b of *K. pneumoniae* by strains carrying pCU1, pCU88 or pCU109.

Plasmid ^c	Marker ^d selected	Frequency(%) of	
		Transfer ^d	Killing ^e
pCU1	Sp	20	80-90
pCU88	Km	10	ND ^f
pCU109	Cm	25	80-90

^aA donor to recipient ratio of 1:10 was used to determine transfer frequencies. Conjugation was at 37°C for 1 h on membrane filter as described in MATERIALS AND METHODS.

^bIn the determination of killing efficiency, a donor to recipient ratio of 20:1 was used. The *K. pneumoniae* survivors were scored on minimal agar (to eliminate the growth of auxotrophic donors) after 48 h of incubation at 37°C.

^cPlasmids were maintained in *E. coli* C600.

^dThe numbers represent the frequency of transfer per 100 donors in a 1 h membrane filter mating at 37°C; it is an average of two experiments.

^eThe number represents the fraction of the *K. pneumoniae* cells in the population survived in comparison to the survivors scored in identical matings with the plasmid-less strain.

^fNot Determined, although in a spot-test it showed comparable killing (as determined by area of clearing) to the killing by a strain carrying pCU1.

TABLE 9.

Determination of Tra^a and Kil^b phenotypes of strains carrying pCU1, pCU101 and pCU105^c

Plasmid ^c	Marker selected for transfer	Tra ^d	Kil ^e
pCU1	Sp	20	10-20 ^f
pCU101	Cm	25	80-100 ^f
pCU105	Cm	20	100

^a Matings were at a donor to recipient ratio of 1:10. Exponentially growing donors and stationary phase recipients were mixed on membrane filters; cells were collected by suction and the filter was incubated on a prewarmed PA plate at 37°C for 1 h; cells were resuspended in saline and dilutions were plated on selective media. Transconjugants colonies were counted after 24-48 h incubation at 37°C.

^b Killing was determined at a donor to recipient ratio of 20:1. The procedure of mating was identical to the one described for transfer. The survivors were scored on glucose minimal agar and counted after 24-48 h.

^c Plasmids were in E. coli C600.

^d The number represents the frequency of transfer "per 100 donors" in two experiments.

^e The number refers to the fraction of K. pneumoniae cells that survived after mating with the donor in comparison to the number of survivors in mating with the plasmid-less strain.

^f The numbers shown are the minimum and maximum number of survivors in five different experiments. In the spot-test (as described in MATERIALS AND METHODS) the strains carrying pCU101 and pCU105 were consistently scored as Kil⁻ while the strain carrying pCU1 was Kil⁺.

Fig. 20. Diagram illustrating the derivation of various pCU1-related plasmids obtained by deletion or molecular cloning experiments from the two transposon Tn5 derivatives of pCU1, pCU29 and pCU88. The triangle shows the site of Tn5 insertion in the plasmids; kilA and kilB, regions required for killing of K. pneumoniae; mob, region required in cis for transfer, Cm, Chloramphenicol, Km, Kanamycin; H, HindIII; B, BamHI; K, KpnI.

sufficient for the Kil^+ phenotype. There are two Tra^+ insertions mapping within 1 Kb of the 3 Kb region of kilB that further reduces this region to 2 Kb (Fig. 12, p109). There are several Tn5 insertions that map within the 3 Kb region on the pCU109 map. However, all the derivatives showed a Kil^+ phenotype. Thus kilA is immediately flanking the leftward end of tra and kilB is located next to the rightward end of tra separated from it by at least 1 Kb.

To determine if kilA and kilB need to be on the same plasmid (i.e. if they are required to be present in cis with respect to one another), a BamHI deletion derivative of pCU29, pCU51 which carries the kilB region was introduced into a strain carrying pCU101 (carries kilA region). The resulting strain did not show any killing ability. To further confirm that pCU51 used in this experiment did indeed carry a potentially functional kilB region, pCU101 and pCU51 were fused at the unique BamHI sites of these two plasmids in vitro by a procedure similar to the one described construction of pCU101. The resulting recombinant designated as pCU114 not only transferred antibiotic markers of both the plasmids but also showed the Kil phenotype. One possible explanation for the failure to observe killing of K. pneumoniae with a strain carrying pCU101 and pCU51 could be due to the

inability of pCU51 to be mobilized. pCU51 does not carry the mob region and is thus unable to be transferred. pCU404, a derivative carrying kilB and mob on pMK2004, a plasmid compatible with pCU101 and which is efficiently mobilized by pCU101 was used in another experiment. The strain carrying pCU101 and pCU404 did show killing thus indicating that the two kil regions kilA and kilB can act in trans but only provided they can be delivered efficiently into the K. pneumoniae strain (Gill, personal communication; see Fig. 20; Table 10).

L) Genetic complementation analysis.

Plasmids pCU1 and pCU109 are intracellularly compatible with one another but have identical tra regions. If two mapped tra mutations that are caused by Tn5 insertion, one on pCU1 and another on pCU109 are placed in the same cell, this can be expected to provide the basis of a reliable genetic complementation test; that is, only if each mutation affected a different Tra-related function would the Tra⁺ phenotype be restored. As a test for the reliability of this rationale and procedure, a recombination-deficient (recA) strain (HB101) carrying a pCU1::Tn5 and a pCU109::Tn5 plasmid was constructed. The Tn5 insert in pCU1 affected pilus synthesis (Tra⁻ Ike^r PRD1^r) and the Tn5 insert in pCU109 did not (Tra⁻ Ike^s PRD1^s). The recombination-deficient strain was used to

a The plasmids were maintained in E. coli C600. The bracket shows that the two plasmids exist independently in the same host. for the description of these plasmids see test.

b All mob plasmids transferred as efficiently as mobilizing plasmid if the mobilizing plasmid was present in the same cell.

c Phenotypes were tested by spot-tests as described in MATERIALS AND METHODS.

TABLE 10.

Determination of functions required
for killing of *Klebsiella pneumoniae*.

Plasmid ^a	Region present on plasmid			phenotype conferred ^c	
	kilA	kilB	mob ^b	Kil	PRD1
pCU1	+	+	+	+	+
pCU101	+	-	+	-	S
pCU51	-	+	-	-	r
pCU101	-	+	+	-	S
pCU51	-	+	-	-	
pCU114	+	+	+	+	S
pCU109	+	+	+	+	S
pCU404	-	+	+	-	r
pCU101	+	-	+	+	S
pCU404	-	+	+	-	
pCU53	+	-	-	-	S
pCU167	-	+	+	-	-
pCU53	+	-	-	-	S
pCU167	-	+	+	-	
pCU57	-	-	+	-	r
pCU58	+	-	+	-	r
pCU167	-	+	+	-	r
pCU57	-	-	+	-	
pCU167	-	+	+	+	S
pCU58	+	-	+	-	

^aGenetic complementation test is described in detail in MATERIALS AND METHODS. All the plasmids were introduced by transformation into *E. coli* HB101. pCU__ derivatives from the horizontal column were introduced first into the strains followed by the pVT__ derivatives. The pCU__ derivatives carried Sp^r and the pVT__ derivatives, Cm^r. All the derivatives were also Km^r (Tn5 specified marker). The recipient strain was GS500 (Rif^r). For transfer, pCU derivatives were selected on RifSp and pVT__ on RifCm. + indicates genetic complementation; - indicates no complementation. In all the cases that showed complementation transfer of the plasmid resulted in a patch of growth on the selective plate and none of the pair showed leaky complementation. Appropriate controls showed no growth on selective plates. A much larger number of strains than is shown here were tested and found to belong to one or the other of the groups indicated. The Table shows representative results.

^bAll pCU__ plasmids are derivatives of the pCU1 carrying Tn5 within tra region. All pVT__ plasmids are derivatives of the pCU109 carrying Tn5 within tra region.

^cLetters represent the assignment to complementation groups.

^dSince no pCU1::Tn5 derivatives in the corresponding region were available for genetic complementation analysis they were initially grouped together. This group (shown in Table by ?) were further subdivided into new groups by the transient heterozygosis technique (For more information on this group see Table 12).

TABLE 11.

Genetic complementation analysis of the
tra region using pCU1::Tn5 and
pCU109::Tn5 derivatives^a.

pCU derivatives ^b							Genetic comple- mentation group ^c
	pCU171	pCU203	pCU157	pCU69	pCU175	pCU185	
pVT derivatives							
pVT80	+	-	-	-	+	+	B
pVT33	+	-	-	-	+	+	B
pVT94	+	-	-	-	+	+	B
pVT58	+	-	-	-	+	+	B
pVT71	+	+	+	+	+	+	? ^d
pVT91	+	+	+	+	+	+	?
pVT118	+	+	+	+	+	+	?
pVT75	+	+	+	+	+	+	?
pVT228	+	+	+	+	+	+	F
pVT170	+	+	+	+	-	-	G
pVT77	+	+	+	+	-	-	G
	A	B	B	B	G	G	

eliminate or greatly reduce recombination between the two plasmids. As can be seen from the results in Table 11, the strain carrying these two plasmids was Tra^+ . Similar tests were then done on all possible pairs of Tn5 mutations. We were particularly interested on pairs the members of which could not be distinguished phenotypically from one another e.g. two mutations both yielding the phenotype $\text{Tra}^- \text{IKer} \text{PRD1}^-$ i.e. affecting pilus biosynthesis. The results of such an analysis (Table 11; Fig. 2f) allowed us to conclude that there were at least five complementation groups in tra, three (traA, B and one more, designated by ? in Table 11) in the pilus region (as defined by physical mapping) and two in the non-pilus region. A mutation within any one of these groups did not complement the transfer-defect of another mutant placed in the same group. Also, all mutations within one complementation group (where >1 such mutation was available) were physically clustered together.

Complementation tests of this type could not be done in a central 9 Kb segment of the tra region because in this segment insertions were available only on one of the two plasmids (pCU109). To circumvent this problem, we first attempted to develop a method to transfer Tn5 insertion from pCU109 to pCU1 by homologous recombination. A pCU109::Tn5 plasmid was cleaved with either KpnI or PvuI neither of which has sites within Tn5 (Jorgensen et al.,

Fig. 21. The position of the transposon Tn5 in the Tra- pCU1::Tn5 and pCU109::Tn5 derivatives. These positions were mapped by using procedures described in the text. Each derivative of pCU1 carrying a specific mapped Tn5 insert has been given a particular "pCU--" designation. Likewise, derivatives of pCU109 have been given a "pVT--" designation. Only the tra region of pCU1 is shown as a line below the pCU1 map and above the pCU109 map. The center bar represents the results of the genetic complementation tests, performed as described in MATERIALS AND METHODS. The boxes represent the complementation units. The letter inside the box is the designation for that tra unit. The solid bar represents mob which overlaps with traG. The area where no Tn5 insertion have been found is enclosed a saw-toothed margins.

1979). The resulting linear forms of plasmid DNA were then used to transform competent cells of a strain carrying pCU1 and several desirable mutations on its chromosome (recB recC sbcB) that greatly reduce the exonuclease activity and selecting for the transposon marker (Km^r) after transforming with linear DNA we may be able to recover some transformants in which the Tn5 was now on pCU1 (at a position homologous to its original position on pCU109). Using this procedure, some Km^r transformants were recovered. Unfortunately, plasmid DNA could not be isolated from these transformants. The reason for this was not investigated any further and the procedure was abandoned because of the success of the analysis using the second procedure, the transient heterozygosis technique.

M) Transient heterozygosis technique.

This procedure has been described in detail in MATERIALS AND METHODS (p80). The principle is that if one Tra- plasmid is introduced by transformation into a strain carrying a second and incompatible plasmid, the two plasmids could be present transiently in the same cell for a sufficient period to express their functions; this could allow complementation if the mutations are in different complementation groups. The results of such analyses are shown in Table 12. The validity of the technique was first established by the observation that its results are

^aThe details of the genetic complementation analysis by the transient heterozygosis technique is described in MATERIALS AND METHODS. In addition to the test plasmid derivatives, appropriate controls were used. The transformed DNA was allowed to express the Tra phenotype and mated en masse with E. coli GS500 on membrane filters for 2 h. The transconjugants were selected on plates containing Rif^{cm}. The phenotypes conferred by strains carrying these plasmids are in Appendix I.

^bAll the plasmids were introduced into E. coli HB101 by transformation.

^cThe amount of donor DNA varied from 2-4 μ g per transformation reaction.

^dThe actual number of colonies on control plates varied from 0 to 40 from one experiment to another. The number of colonies obtained on plates from the complementation tests between isogenic plasmids were used as the controls. In cases where no complementation was seen, the number of colonies found were within the above mentioned limit.

^eNot Determined.

^fThree pluses show that the number of transconjugants obtained were at least 10-fold or higher. The actual number varied from 50 to 800.

^gTwo pluses indicates that the number of transconjugants were 2 to 10 fold higher than the number obtained on the control plates. The actual number varied from 50 to 200 colonies. In only one case (pVT96 and pVT91) was the increase in number of transconjugants 1.5 fold. This increase was however obtained consistently in 2 experiments. This was therefore considered to be a case of weak complementation. There was only a single mutation in this class.

TABLE 12.

Genetic complementation by the transformation mediated transient heterozygosis technique^a.

Donor DNAC	Recipient ^b			
	pVT94	pVT91	pVT118	pVT75
pVT80	-d	ND ^e	ND	ND
pVT94	-	-	ND	ND
pVT63	ND	-	ND	+++ ^f
pVT91	ND	-	ND	+++
pVT71	+++	-	ND	+++
pVT96	++ ^g	+	+++	+++
pVT118	+++	+++	-	+++
pVT106	ND	++	-	+++
pVT69	ND	+++	-	+++
pVT82	ND	ND	-	ND
pVT97	ND	++	+++	++
pVT64	ND	ND	+++	-
pVT54	ND	ND	++	-
pVT95	ND	++	++	ND
pVT75	+++	+++	+++	-
pVT61	ND	ND	ND	-
pVT228	+++	ND	ND	ND
pVT119	ND	ND	+++	ND
pVT144	ND	+++	ND	ND

in agreement with the results of the technique using the non-transient heterozygotes (i.e. pCU1::Tn5 and pCU109::Tn5); that is, the same five complementation groups defined by the first technique were also revealed by the second. Furthermore, the latter technique has revealed three more complementation groups in the central 9 Kb segment for a total of eight complementation units in the tra region of pCU1 (Table 11 and 12; Fig. 21). Three comments on these results are in order:

- (a) For IKe^r PRD1^r mutants mapping to the pilus region (Fig. 17), complementation leading to sensitivity to these phages was always accompanied by complementation to transfer proficiency;
- (b) Complementation between mutations in the region shown as traD and mutations in only traC but not traE was weak (1.5 fold) but consistent;
- (c) The eight complementation units that are defined by this analysis must be considered to be a minimum estimate because there are 1 to 2 Kb segments of the tra region between some complementation groups in which no insertions were obtained (Fig. 21) thus preventing analyses of these regions.

DISCUSSION

In this study, the tra functions of plasmid pCU1 have been localized to a 15 Kb region of the 39 Kb plasmid. In other conjugative plasmids including those that determine different conjugative systems, a major segment of each plasmid is occupied by its tra region. Plasmid-pCU1 does not differ from others in this respect. The tra region of pCU1 was analysed further to determine if it forms a single continuous region as in the classical conjugative plasmid F (Willettts and Skurray, 1980) or whether it has dispersed tra regions as in P and W group plasmids (Barth, 1979; Tait et al., 1982). To do this, we attempted to saturate the parental plasmid and a clone carrying the pCU1-tra region by mutagenesis with the transposon Tn5. Despite isolating and mapping 117 insertions within tra, we were unable to saturate the region. There are two regions of DNA 1.3 Kb and 2 Kb in size that are located within tra where none of the insertions that were isolated could be mapped. As a result, it is not known whether or not these regions are involved in tra functions. If not, the pCU1-tra functions would be distributed in a maximum of three distinct regions. Other N group plasmids that have been studied are R46, its deletion derivative pKM101 and N3. A derivative carrying a 22.5 Kb region of R46 when cloned into a non-conjugative plasmid, pBR322, was found to be self-transmissible (Brown and Willettts, 1981). Although

R46-tra has not been analyzed further, the tra region of its derivative pKM101 has been studied using transposon mutagenesis and cloning (Langer et al., 1981). Tra functions were assigned to a minimum of a 15 Kb region of this plasmid. In a study of the relationship of an endonuclease encoded by this plasmid to Tra and related functions, Winans and Walker (1983) have localized a 0.8 Kb region responsible for the production of this endonuclease. This region called nuc, mapped within the 15 Kb tra region. Among other possibilities, it was suspected that the endonuclease activity may be involved in either nicking of DNA at oriT (origin of plasmid transfer) or in the killing of Klebsiella pneumoniae. Among 250 transposon Tn5 insertions mapped on pKM101, none had any insertion in the nuc region; however, one transposon derivative was isolated in a clone which carried the insertion in the nuc region. When this transposon was exchanged with its homologous region on pKM101, the resultant derivative showed a Tra⁺ and Kil⁺ phenotype. Thus this insertion in pKM101 did not affect the Tra and Kil phenotypes and it could be concluded that pKM101-tra is dispersed into at least two distinct regions. These two N group plasmids, R46 (or pKM101) and pCU1 have different origins. R46 was isolated from a Salmonella strain in the United Kingdom (Drabble and Stocker, 1968) and pCU1 was isolated from a Salmonella

strain in Eastern Canada (Khatoun and Iyer, 1972; Konarska-Kozłowska and Iyer, 1981a). A comparison of the tra regions of these two plasmids reveals that the order and number of sites for several restriction endonucleases that cleave the DNA in this region are the same (see Fig. 22). This indicates that the two plasmids are similar. The other N group plasmid, N3 has been studied by Ando and Arai (1981), who have constructed a map of this plasmid using four restriction enzymes. The tra region of this plasmid has been localized within the largest SalI fragment (22.6 Kb). The organization of tra within this fragment has not been reported; however the absence of a SalI site within the tra region of N3 and its presence on pCU1 (and R46) indicates that the tra regions of N3 is different from that of pCU1 or R46 (pKM101). A large number of N-group plasmids have been shown to possess endonuclease activity. However, pCU1 has not been tested for this activity. The nuc gene of pKM101 is located close to the 2.0 Kb region of pCU1 where no Tn5 insertions were mapped. Thus, the possibility that tra of pCU1 is dispersed in two or three regions remains open. Plasmids of two other groups, P and W also show dispersed tra regions (Barth, 1979; Lanka and Barth, 1981; Tait *et al.*, 1982; see REVIEW OF THE LITERATURE). The reasons for not obtaining any insertion within the two small regions of pCU1 are not clear and any interpretation of it would be

Fig. 22. A comparison of the distribution of restriction sites in the transfer regions of the N group plasmid pCU1 and R46 (pKM101). nuc, endonuclease; ? denotes regions of pCU1 where no insertions mapped.

Abbreviations for restriction sites: H, HindIII, Sa, Sall; K, KpnI, Sm, SmaI, Bg, BglII; P, PstI.

TRA

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speculative. It is possible, for example, that one or both regions may be needed to suppress the activity of a gene or genes within tra that is detrimental to the host cells. There are instances cited in the literature where such regions have been found. In plasmid RP4, there are at least four distinct genes whose existence on the plasmid was found to be lethal to the host. This phenomenon is different from the killing of K. pneumoniae by N group plasmids, which requires conjugation. These genes, also called kil genes and not to be confused with the Klebsiella kil region could be localized by using another set of genes called the kor genes (for kill override). Each kor gene was able to override only its specific kil gene (Figurski et al., 1982; Young et al., 1984). If any one of the kor genes was absent or mutated, the plasmid had also to be mutated in a corresponding kil gene without which the E. coli cells would not survive.

A subset within tra of pCU1 was sufficient for N-pilus-specific sensitivity to bacteriophages IKE and PRD1. None of the mutants that showed resistance to IKE was transfer proficient indicating that the N-pili to which this phage attaches (Bradley, 1980) are absolutely necessary for the conjugal transfer of these plasmids. Three insertions that abolished the sensitivity to phage PRD1 were Tra⁺. It is possible that the region defined by these three insertions is not needed for N pilus

production per se but is needed to modify the pilus during or after it is formed since all three mutants showed sensitivity to Ike. Ike and a phage, PR4 that is believed to be identical to PRD1 have been used in electron microscopic studies of phage binding to bacterial cells. Both Ike and PR4 (and therefore presumably PRD1) were reported to bind to the tips of N pili (Bradley, 1980). However, N pili attached to bacterial cells have been difficult to visualize. In the literature, there is only one instance showing N-pili attached to bacterial cells. In this same study however, electronmicrographs are shown where Ike is attached to the tips of free pili detached from cells. In another study (Brodt et al., 1974), phage Ike was found attached directly to the cell surface. This observation was accommodated (Bradley, 1980) by suggesting that the N pilus had retracted into the cell following attachment to the N pilus tip. Phage PR4 (indistinguishable from PRD1) was also shown to be attached to the tips of such free N-pili only. Thus, there is still some uncertainty concerning the conclusion that both of these phages attach to pilus tips when these pili are on the cell surface. There is also no information concerning the precise role of the N pilus in conjugation.

It is clear from the present study that PRD1-sensitivity can be altered mutationally without

altering IKE-sensitivity or the Tra phenotype. Several explanations for this observation could be postulated but all of these would need further study.

a) the binding site for PRD1 may be different from that of IKE (even though both phages are seen morphologically attached to the tips of free pili).

b) binding of PRD1 occurs at the same site as that of IKE but in the mutant any of several further steps are aborted for PRD1 but not for IKE.

In any case, the product of this gene may not grossly alter the N pilus and particularly the N pilus tip because the strain remains sensitive to phage IKE, a phage that also attaches to the tip. Since the mutations giving this phenotype could be complemented in trans, it is likely that the region codes for a diffusible product. All three mutations also affected the Kil phenotype. However, this does not rule out the possibility that there are two or more genes in the kilA region both of which are co-transcribed. Whether all three mutations affect only one gene or whether a mutation in one is polar on another is not known. The 10.2 Kb region required for pilus production does not include this additional 1 Kb region required for the sensitivity of cells carrying pCU1 to PRD1. In other tra systems that have been studied it is known that a significant portion (60-70% of tra) is required for the production of functional pili and the.

genes responsible for it are usually tightly clustered. This study shows that the tra system of pCU1 does not differ from other tra systems in this respect.

Using molecular cloning, we have localized on pCU1 a region that is required to be present in cis on the plasmid DNA being transferred. This region called mob is within a 1.2 Kb length at one end of the tra. Our attempt to obtain a cis-dominant mutation within this region failed. This may be due to the fact that this region is much smaller than 1.2 Kb.

As a contribution to the understanding of the genetic organization of tra, we used a large number of Tn5 insertions on the parental plasmid pCU1 and on its derivative carrying tra and related functions. Eight complementation units were defined within the tra region. The transposon Tn5 is known to have transcriptional termination sites and insertion of it are known to be polar if inserted in other operons. Therefore, it is very likely that the eight complementation groups defined represent the minimum number of tra transcriptional units. The direction of transcription or the number of genes within any particular transcriptional unit have not been defined.

All N group plasmids that have been tested confer on their E. coli host cells the capacity to kill a Klebsiella pneumoniae strain upon mating with the latter.

This phenotype must be determined by a gene or genes that are conserved by N group plasmids. Two such regions are the maintenance region and the tra region. Rodríguez (1981) has demonstrated that conditions promoting efficient conjugation are necessary for this killing. It therefore seemed likely that the gene(s) required for killing would be localized within tra. However, this study has shown that a cloned region that conferred full conjugal ability is unable to show the Kil phenotype. By transposon mutagenesis and molecular cloning, we have shown that there are two kil regions (kilA and kilB) that are located at the ends of tra but does not form part of it. Both regions are needed for the expression of the Kil phenotype. The kilB region could not be inactivated and was localized on the basis of cloning. Two other groups of plasmids, P and W, that show killing ability but to a lesser degree than N also share another common phenotype, sensitivity to phage PRD1. This study has shown that there is a relationship between these two phenotypes. The study shows that for killing to occur both the kil genes are needed to be transferred into the recipient K. pneumoniae. However, kilA and kilB can act when they are on separate DNA molecules. The mechanism of killing remains unknown. A model has been proposed by Singleton (1983). This model was based on our observations that killing is observed only under conditions that permits the

efficient transfer of N plasmids. N pili are short and pointed ("thorn-like"), as opposed to long and flexible pili specified by F and F-like plasmids. Singleton proposes that these pili are pressed against the recipient cell-wall during mating, that this may rupture the recipient cell envelope thus resulting in killing. The following observations rule out such a mechanism as being sufficient: i) some mutants strains which produced N pili did not kill K. pneumoniae (e.g. strains carrying pCU53 or pCU102); ii) not all strains carrying Tra⁺ derivatives of pCU1 that produce pili and conjugate efficiently showed killing (e.g. strains carrying pCU101 or pCU167). In all experiments where a plasmid or combination of plasmids showed the Kil phenotype, a plasmid carrying a functional pCU1-tra system was co-transferred. Therefore, the possibility of interactions involving gene(s) within tra in addition to kilA and kilB is not ruled out.

Fig 23 is a diagrammatic summary of all the results presented in this study.

Fig. 23. A physical and genetic map of pCU1. The restriction map is based on Konarska-Kozłowska et al. (1983). The numbers inside the circle are the Kb coordinates of pCU1. The limits of the transfer region and of different sub-sets of it are based on data presented in this study. The letters inside the boxed area refer to the genetic complementation group. The saw-toothed line enclose areas of uncertainty. PIL denotes N pilus region; Mob, a region required in cis for transfer; KilA and KilB are the regions needed for the killing of Klebsiella pneumoniae; Kim, the region conferring immunity to killing (a phenotype conferred in Klebsiella; S. Gill, M.Sc. thesis, in preparation); Mnt, pCU1 maintenance region; Sp^r, Sm^r, Ap^r, resistance to Spectinomycin, Streptomycin and Ampicillin respectively. The abbreviations used for restriction endonucleases are: AI, A_{va}I; BI, B_{am}HI; BgII, B_{gl}II; BsII, B_{st}EII; EI, E_{co}RI; HI, H_{ind}III, HpI, H_{pa}I; KI, K_{pn}I; P_{vi}I, P_{vu}I; PI, P_{st}I; SI, S_{al}I; SmI, S_{ma}I.

APPENDIX I

pCU188	29.6	same as pCU164	
pCU243	30.0	same as pCU164	
pCU653	30.2	same as pCU164	
pCU99	30.5	same as pCU164	
pCU192	30.6	same as pCU164	
pCU187	30.7	same as pCU164	
pCU171	32.2	- r r - r r r	A
pCU203	33.2	same as pCU171	B
pCU176	33.4	same as pCU171	B
pCU77	33.6	same as pCU171	B
pCU183	33.7	same as pCU171	B
pCU200	33.9	same as pCU171	B
pCU184	34.0	same as pCU171	B
pCU153	34.2	same as pCU171	B
pCU157	34.5	same as pCU171	B
pCU159	34.7	same as pCU171	B
pCU244	35.0	same as pCU171	B
pCU202	35.5	same as pCU171	B
pCU169	36.0	same as pCU171	B
pCU175	6.4	- s s - r r r	B
pCU62	6.4	same as pCU175	H
pCU85	7.0	same as pCU175	H
pCU67	7.2	same as pCU175	H
pCU156	7.3	same as pCU175	H
pCU178	7.5	same as pCU175	H
pCU106	7.8	same as pCU175	H
pCU185	8.0	same as pCU175	H

b. pCU109::Tn5 derivatives

Plasmid (design- ation)	Map position	Phenotype conferred Tra Ike PRD1 Kil Cm	Genetic complemen- tation group
pVT80	32.8	- r r - r	B
pVT81	33.81	- r r - r	B
pVT33	33.3	same as pVT80	B
pVT70	33.5	same as pVT80	B
pVT94	33.7	same as pVT80	B
pVT84	33.9	same as pVT80	B
pVT83	34.2	same as pVT80	B
pVT57	34.6	same as pVT80	B
pVT58	34.9	same as pVT80	B
pVT73	36.3	same as pVT80	C
pVT71	36.5	same as pVT80	C
pVT63	36.6	same as pVT80	C
pVT91	36.9	same as pVT80	C
pVT96	38.1	same as pVT80	D
pVT118	38.8	same as pVT80	E
pVT106	00.3	same as pVT80	E
pVT69	00.5	same as pVT80	E
pVT82	00.7	same as pVT80	E
pVT53	1.0	same as pVT80	E
pVT97	1.2	same as pVT80	F
pVT64	1.4	same as pVT80	F
pVT54	1.6	same as pVT80	F
pVT95	2.0	same as pVT80	F
pVT75	2.2	same as pVT80	F
pVT61	2.6	same as pVT80	F

pVT213	3.1	- s s - r	G
pVT228	5.1	same as pVT80	H
pVT170	5.3	same as pVT213	H
pVT160	5.5	same as pVT213	H
pVT173	5.5	same as pVT213	H
pVT161	5.5	same as pVT213	H
pVT200	5.8	same as pVT213	H
pVT205	6.1	same as pVT213	H
pVT144	6.3	same as pVT213	H
pVT149	6.3	same as pVT213	H
pVT151	6.5	same as pVT213	H
pVT217	6.8	same as pVT213	H
pVT150	7.1	same as pVT213	H
pVT119	7.3	same as pVT213	H
pVT108	7.5	same as pVT213	H
pVT214	7.7	same as pVT213	H
pVT77	7.9	same as pVT213	H

^aTra⁺ pCU109::Tn5 derivatives are not listed here. For their map position see Fig. 15, p118. They were designated as pSV____. All of them showed Tra⁺ Ike^s PRD1^s Kil⁺ phenotypes.

^bPlasmids were maintained in *E. coli* C600 or HB101.

^cMap position corresponds to the Kb coordinates on the pCU1 map (see Fig. 22).

^dPhenotypes were checked as described in MATERIALS AND METHODS. Tra, conjugal transfer; Ike and PRD1, bacteriophages Ike and PRD1; Kil, killing of *Klebsiella pneumoniae* strain; Ap, Ampicillin; Sm, Streptomycin; Sp, Spectinomycin; Km, Kanamycin; + or -, presence or absence of phenotype; r, resistance; s, sensitivity.

^ethe letter refers to the tra transcriptional unit as described in the text (also see Fig. 21).

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