1

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service

Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

NOTICE

The quality of this microform is heavily dependent upon the quality of the original thesis submitted for microfilming. Every effort has been made to ensure the highest quality of reproduction possible.

If pages are missing, contact the university which granted the degree.

Some pages may have indistinct print especially if the original pages were typed with a poor typewriter ribbon or if the university sent us an inferior photocopy.

Reproduction in full or in part of this microform is governed by the Canadian Copyright Act, R.S.C. 1970, c. C-30, and subsequent amendments.

AVIS

La qualité de cette microforme dépend grandement de la qualité de la thèse soumise au microfilmage. Nous avons tout fait pour assurer une qualité supérieure de reproduction.

S'il manque des pages, veuillez communiquer avec l'université qui a conféré le grade.

La qualité d'impression de certaines pages peut laisser à désirer, surtout si les pages originales ont été dactylographiées à l'aide d'un ruban usé ou si l'université nous a fait parvenir une photocopie de qualité inférieure.

La reproduction, même partielle, de cette microforme est soumise à la Loi canadienne sur le droit d'auteur, SRC 1970, c. C-30, et ses amendements subséquents.

**SEQUENCE AND DELETION ANALYSIS OF THE *ORIT* REGION
OF THE N CONJUGATIVE TRANSFER SYSTEM OF PLASMID pCU1
AND ITS SPECIFICITY FOR *MOB* FUNCTIONS OF OTHER N TRANSFER SYSTEMS.**

by

E. Suzanne Paterson, B.Sc.

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

**Master of Science
Department of Biology**

**Carleton University
Ottawa, Ontario**

March 1

copyright

1991, Suzanne Paterson

National Library
of Canada

Bibliothèque nationale
du Canada

Canadian Theses Service Service des thèses canadiennes

Ottawa, Canada
K1A 0N4

The author has granted an irrevocable non-exclusive licence allowing the National Library of Canada to reproduce, loan, distribute or sell copies of his/her thesis by any means and in any form or format, making this thesis available to interested persons.

The author retains ownership of the copyright in his/her thesis. Neither the thesis nor substantial extracts from it may be printed or otherwise reproduced without his/her permission.

L'auteur a accordé une licence irrévocable et non exclusive permettant à la Bibliothèque nationale du Canada de reproduire, prêter, distribuer ou vendre des copies de sa thèse de quelque manière et sous quelque forme que ce soit pour mettre des exemplaires de cette thèse à la disposition des personnes intéressées.

L'auteur conserve la propriété du droit d'auteur qui protège sa thèse. Ni la thèse ni des extraits substantiels de celle-ci ne doivent être imprimés ou autrement reproduits sans son autorisation.

ISBN 0-315-68920-X

Canada

Carleton University
Ottawa, Canada K1S 5B6

Thesis contains black & white
photographs &/or explanatory tables
which when microfilmed may lose their
significance. The hardcopy of the
thesis is available upon request from
Carleton University Library.

The undersigned recommend to the Faculty of Graduate Studies
and Research acceptance of the thesis

SEQUENCE AND DELETION ANALYSIS OF THE *ORIT* REGION
OF THE N CONJUGATIVE TRANSFER SYSTEM OF PLASMID pCU1
AND ITS SPECIFICITY FOR *MOB* FUNCTIONS OF OTHER N TRANSFER SYSTEMS.

submitted by E. Suzanne Paterson, B.Sc.

in partial fulfilment of the requirements for
the degree Master of Science

Thesis Supervisor

Chair, Department Biology

Carleton University

April, 1991

ABSTRACT

The *oriT* region of the conjugative, IncN plasmid pCU1 has been localized to a 669 bp sequence extending from pCU1 co-ordinate 8.48-9.15 Kb. The nucleotide sequence of this region was determined. Prominent structural features include one set of thirteen, 11 bp direct repeats, a second set of two 14 bp direct repeats, six different inverted repeat sequences ranging from 6 to 10 bp in size and an 81 bp sequence which is 79% AT residues. A pCU1 relaxation-complex was not observed; however, an *in vivo* nicking event was localized to a 65 bp sequence within the 669 bp *oriT* sequence. Mobilization experiments between the pCU1 *oriT* region and several conjugative plasmids encoding N type *tra* systems demonstrated a plasmid specificity for *mob* functions. This specificity was encoded by the region lying clockwise of the *Bgl*II site at 3.3 on the pCU1 map.

ACKNOWLEDGEMENTS

First and foremost I wish to thank Dr. Iyer for his support and guidance in the completion of this project. I appreciate his patience in allowing me to carry out my research on a part time basis.

I would also like to thank Dr. Carmody and Dr. Hickey for serving on my Research Committee. Their suggestions and advice through out the research process was greatly appreciated.

Over the years there have been several people in Dr. Iyer's research lab whose comments, advice and suggestions have greatly assisted me in my endeavours. In particular I would like to thank John Nash for several years of advice as well as for reading the first draft of this thesis. Thanks also go to Vinay Thatte who always had time for questions and whose plasmid DNA I borrowed frequently.

Finally, but certainly not least, I wish to thank my husband Tony for his constant support and encouragement through out this process.

This research was sponsored by a grant to Dr. Iyer from the Medical Research Council of Canada and by an equipment grant from the Natural Sciences and Engineering Research Council of Canada.

TABLE OF CONTENTS

ACCEPTANCE SHEET	ii
ABSTRACT	iii
ACKNOWLEDGEMENTS	iv
TABLE OF CONTENTS	v
LIST OF TABLES	viii
LIST OF FIGURES	ix
LIST OF ABBREVIATIONS	xi
1. INTRODUCTION	7
2. LITERATURE REVIEW	7
2.1. Components of conjugal DNA metabolism	7
2.1.1. <i>oriT</i>	7
a) General structure and function	7
b) DNA sequence within the <i>oriT</i> region	9
2.1.2. Relaxation complex	13
a) Relaxation complex of ColE1	13
b) Relaxation in F like plasmids	15
c) Relaxation in other plasmids	16
2.1.3. Other functions involved in conjugal DNA metabolism	17
2.2. Mobilization	18
2.2.1. Mobilization by donation	18
a) General features of donation	18
b) Interaction between the <i>mob</i> functions of non-conjugative plasmids and the <i>tra</i> functions of conjugative plasmids	19

c) Interaction between the <i>mob</i> functions of different conjugative plasmids encoding similar transfer systems	22
2.2.2. Mobilization by conduction	24
a) General features of conduction	24
b) Conduction involving homologous recombination	24
c) Conduction involving site-specific recombination	25
2.3. The N transfer system	26
3. METHODS AND MATERIALS	29
4. RESULTS AND DISCUSSION	48
4.1. Localization of <i>oriT</i>	48
4.1.1. Redefinition of the right hand limit of <i>tra</i>	48
4.1.2. Subcloning the <i>oriT</i> region from pVT30: construction of pSP1	54
4.1.3. Construction of pSP3	57
4.1.4. Bal31 deletion mutagenesis of pSP3	60
4.1.5. Construction of deletions within pBP1 and pBE60	68
4.1.6. Mobilization of <i>oriT</i> derivatives	75
4.2. Sequence analysis of the <i>oriT</i> region	80
4.2.1. Cloning into M13	81
4.2.2. Cloning into pIB176	81
4.2.3. Single stranded DNA sequencing	82
4.3. Attempts to identify a pCU1 relaxation complex	93
4.4. Determination of donation vs conduction as the mode of mobilization	98
4.4.1. Replica plating transconjugants to determine co-	

transfer of conjugative and non-conjugative plasmids	98
4.4.2. Eckhardt gel electrophoresis of transconjugants	101
4.4.3. Restriction digest analysis of pBP11 and pSP14 derivative	
DNA altered during conjugal transfer	106
4.4.4. Restriction analysis of pSP20 and pSP21 derivatives altered	
during conjugation	115
4.5. Identification of plasmid specific <i>trans</i> acting <i>tra</i> functions	116
4.5.1. Specificity for <i>mob</i> functions among N type transfer systems	116
4.5.2. Localizing the pCU1 specific <i>mob</i> functions	118
a) Construction of deletions into <i>mob</i>	121
b) Mobilization experiments on the <i>mob</i> deletion constructs	124
4.5.3. Complementation of Tra ⁻ pKM101::Tn5 insertion mutants	
by pMUR plasmids	128
4.5.4. Complementation of pKM101 <i>traJ</i> mutations by conjugative	
plasmids encoding different <i>tra</i> systems	130
5. FURTHER DISCUSSION	132
6. CONCLUSIONS	144
7. REFERENCES	145

LIST OF TABLES

TABLE	DESCRIPTION	PAGE
3.1.	<i>E. coli</i> strains used in this study	30
3.2.	Bacteriophages used in this study	30
3.3.	Plasmids used in this study	31
4.1.	Mobilization frequency of pCU1 <i>oriT</i> derivatives by conjugative plasmid pCU101	76
4.2.	Mobilization frequency of pCU1 <i>oriT</i> derivatives by conjugative plasmids pCU1 or pCU401	77
4.3.	Mobilization of pBP1, pCU57 and pCU56/pCU404 by IncN plasmids	120
4.4.	Mobilization of pCU57, pCU56, pBP1, pBGL and their derivatives by pCU1 and the pMUR:: <i>Tn10</i> derivatives pSP274 and pSP545	125
4.5.	Mobilization of pMK101:: <i>Tn5</i> mutants by the pMUR:: <i>Tn10</i> derivatives pSP274 and pSP545	129
4.6.	Mobilization of pMK101 <i>traH1143</i> and pMK101 <i>traJ35</i> by different transfer systems	131

LIST OF FIGURES

FIGURE	DESCRIPTION	PAGE
4.1.	Plasmid derivatives of pCU1 used in this study	49
4.2.	Comparison of <i>Hpa</i> I restriction patterns of pCU29	52
4.3.	Construction of pSP1 and pSP3	55
4.4.	Comparison of pSP1 and pSP3 restriction patterns	58
4.5.	Detailed restriction map of pSP3	61
4.6.	Gel electrophoresis of pSP3 <i>Bal</i> 31 deletion derivatives	64
4.7.	Schematic representation of pSP3 <i>Bal</i> 31 deletion derivatives and their corresponding mobilization frequencies by pCU101	66
4.8.	Construction of deletions within pBP1	69
4.9.	Construction of deletions in pBE60	71
4.10.	Expanded pCU1 <i>oriT</i> region showing the extent of deletions into each <i>oriT</i> derivative	73
4.11.	Analysis of variance of mobilization frequency means.	79
4.12.	Strategy for sequencing the pCU1 <i>oriT</i> region	83
4.13.	pCU1 <i>oriT</i> region sequence	86
4.14.	Homology between sequences of the pCU1 <i>oriT</i> region and the <i>oriT</i> regions of ColE1, CloDF13 and pSC101	90
4.15a-c.	Relaxation of pBRK4013 and pCU1 derivatives	94
4.16a-c.	Eckhardt gel electrophoresis of transconjugants resulting from matings between C600NaI ^r and HB101/pCU101/pBE60 HB101/pCU101/pMK2004 and HB101/pCU101/pBP11	102

4.17.	<i>Bam</i> HI+ <i>Hind</i> III restriction digest of pBP11 derivatives altered during mobilization by pCU101	108
4.18.	Proposed co-integrate structure between pCU101 and pBP11 leading to the formation of the three altered pBP11 derivatives found in transconjugants from matings between C60uNal and HB101/pCU101/pBP11	112
4.19.	The <i>mob</i> region of pCU1 and pKM101	122
5.1.	Summary diagram of the <i>oriT</i> region of pCU1	140

LIST OF ABBREVIATIONS

+/-	presence or absence of phenotype
<	less than
>	greater than
A	Adenine
Ap	Ampicillin
bom	basis of mobility
bp	base pair(s)
C	Cytosine
°C	degrees celsius
cfu	colony forming units
Cm	Chloramphenicol
CsCl	Cesium chloride
CSSR	Conservative site-specific recombination
dNTP	deoxynucleotide triphosphate
dH₂O	deionized water
DNA	deoxyribonucleic acid
DCDS	Complementary DNA strand synthesis in donor
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethyleneglycol-bis-(β-amino-ethyl ether)N,N'-tetraacetic acid
F'	F plasmid carrying chromosomal DNA
G	Guanine
hr	hour(s)

Inc	Incompatability
IHF	Integrative host factor
<i>int</i>	integrative host factor gene
IPTG	Isopropyl-β-D-thiogalactopyranoside
IS	Insertion sequence
LB	Luria-Bertania broth
LBA	Luria-Bertania agar
Km	Kanamycin
Kb	Kilobase(s)
mamp	milliamp(s)
mM	millimolar
M	molar
min	minute(s)
ml	millilitre(s)
mg	milligram(s)
MgCl₂	Magnesium chloride
Mob	mobilization
NaCl	Sodium chloride
Nal	Nalidixic acid
<i>nic</i>	site of strand specific nicking within the oriT
<i>oriT</i>	origin of transfer
<i>oriV</i>	origin of vegetative replication
PA	Penassay agar
PB	Penassay broth

PEG	Polyethylene glycol
pfu	plaque forming units
r	resistant
rec	recombination gene
Rif	Rifampicin
RCDS	Complementary DNA strand synthesis in recipient
RFM	revolutions per minute
SDS	Sodium dodecyl sulfate
s	sensitive
ss	single-stranded
sec	second(s)
Sm	Streptomycin
Sp	Spectinomycin
STET	Sucrose Tris EDTA Triton-X
T	Thymine
TA	Tris acetate restriction buffer
TAE	Tris acetate EDTA electrophoresis buffer
TBE	Tris borate EDTA electrophoresis buffer
Tc	Tetracycline
TE	Tris EDTA
Tn	transposon
tra	transfer gene/region
UV	ultraviolet
w/v	weight per volume

X-gal 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

YT Yeast tryptone broth

μ Ci microcurri(es)

μ g microgram(s)

μ l microlitre(s)

μ M micromolar

1. INTRODUCTION

Bacterial conjugation can be defined as the transfer of DNA from one bacterial cell (donor) to another (recipient) during cell to cell contact. (Willetts and Skurray, 1980). Conjugal DNA transfer is a natural phenomenon common to both Gram negative and Gram positive bacteria. Of the three modes of DNA transfer available to bacteria, namely transformation, transduction and conjugation, conjugation is the most efficient. As such it is a useful tool to the molecular geneticist for the transfer of DNA between different bacterial strains.

To date, conjugative functions in Gram negative bacteria have always been associated with extrachromosomal elements called 'conjugative' or 'self transmissible' plasmids. The genes responsible for conjugative functions are referred to as transfer (*tra*) genes. Conjugative plasmids are relatively large (from 25 to over 100 Kb) and the region encoding *tra* functions can constitute one quarter to one third of the plasmid.

The best-studied conjugative plasmid is the F plasmid which was isolated from the Gram negative bacterium *Escherichia coli*. Much of what is known about bacterial conjugation in Gram negative bacteria comes from the study of the F plasmid. The conjugation system of the F plasmid has been reviewed by Ippen-Ihler and Minkley (1986) and Willetts and Skurray (1980).

Since the discovery of the F plasmid, numerous other conjugative plasmids have been isolated from Gram negative bacteria. Attempts made to classify these new plasmids on the basis of their conjugative characteristics were quickly abandoned in favour of the more fundamental function of replication and stability

within the host. As a result the plasmids were grouped into incompatibility groups (Inc) according to the inability of members of the same incompatibility group to be stably maintained in the same cell (Datta, 1974). Fortunately, similarities in replication correlate well with similarities in conjugative functions such that plasmids belonging to one incompatibility group generally encode similar transfer systems. Similarity between transfer systems is determined by DNA homology, the ability of one conjugative plasmid to complement transfer deficient mutations in another, morphological and serological similarities between their conjugative appendages (pili) and susceptibility to a small but diverse group of male specific bacteriophages (Lawn *et al.*, 1967).

Unlike the F *tra* system, relatively little is known about the transfer systems of other Gram negative conjugative plasmids. To date, only the P, I, N and to some extent the W transfer systems have been investigated in any detail. Although these other transfer systems demonstrate wide genetic variation, they have been shown to possess components which are functionally similar to those of the F transfer system. Therefore while it is generally held that all Gram negative bacterial systems function in a similar manner, further research is required to determine the extent of the similarity.

Conjugation in Gram negative bacteria consists of two major events, i) the formation of stable mating pairs or aggregates; and ii) conjugal DNA metabolism (Clark and Warren, 1979). Both of these events or components of conjugation are encoded by several *tra* genes. Additional *tra* genes have been shown to be involved in minor conjugal functions such as surface exclusion and fertility inhibition, which while not essential for transfer, affect conjugal transfer by either preventing the

introduction of a plasmid into a cell already carrying that plasmid or a closely related plasmid (Willetts and Skurray, 1980) or by inhibiting the transfer of a non-related plasmid within the same cell (Olsen and Shipley, 1975, Tanimoto and Iino, 1983, Pinney and Smith, 1974, Winans and Walker, 1985). In some cases within the F and H transfer systems, one or more of the *tra* genes are involved in the regulation of the other *tra* genes (Willetts and Skurray, 1980).

The formation of conjugal mating pairs requires the production of special cell appendages called sex or conjugative pili (Ippen-Ihler and Valentine, 1967). Studies by Bradley (1980) have distinguished three different morphological types of pili: long, thin and flexible, long, thick and flexible and short and rigid. Within these three morphological groups, pili can be further subdivided serologically and on the basis of their susceptibility to male specific bacteriophages (Bradley, 1980, and 1981).

There is some evidence to suggest that the F type pili bring the mating cells into direct wall to wall contact by retracting into the donor cell following contact with the recipient cell (Jacobson, 1972). Beyond the formation of mating pairs the role of the pili is unknown. Whether the pili act as a hollow tunnel or port through which DNA can pass or simply as a connecting element by which the cells are brought into contact allowing for a joining of the cell membrane has not yet been established (Panicker and Minkley, 1985). For the F plasmid, subsequent stabilization of mating aggregates has been reported to be encoded by two additional genes, *traN* and *traG* (Willetts and Skurray, 1980).

Conjugal DNA metabolism refers to all of the functions involved in processing the DNA prior to and immediately following the actual transfer. In the case of the F plasmid, conjugal DNA transfer begins with the nicking of one strand of plasmid DNA

at a specific site. The nicked strand is then unwound and the resultant linear single-stranded DNA passes from the donor cell into the recipient cell, such that the *tra* genes enter last. Within the recipient cell a complementary strand is synthesized and the linear double stranded DNA is recircularized. A complementary strand is also synthesized in the donor cell from the remaining strand. Willetts and Wilkins (1984) have reviewed the information available on DNA processing in Gram negative bacteria.

Most of the conjugative functions are *trans* acting. The only *cis* acting *tra* function is the site at which the single strand of DNA is nicked and from which transfer originates: the origin of transfer (*oriT*). The *oriT* is considered to be the focal point of conjugal DNA processing. It is the site at which several *trans* acting *tra* functions must interact in order to facilitate nicking in the donor cell as well as strand replacement in the donor and recipient cells. As the leading end of the single stranded, linear DNA it must also interact with the cell membrane in order for the DNA to be passed from donor to recipient cell.

In addition to conjugative plasmids, there exists a group of much smaller plasmids (less than 10 Kb) which are unable to promote their own transfer during conjugation. These so-called non-conjugative plasmids can however be transferred in the presence a self-transmissible plasmids by a process known as mobilization.

Mobilization can occur by two different processes: donation and conduction (Clark and Warren, 1979). A non-conjugative plasmid is said to be donated if it is transferred without being physically joined to the helper conjugative plasmid such that the two plasmids transfer as separate DNA elements. Non-conjugative plasmids which can be donated carry an *oriT* as well as some *trans* acting functions

necessary for their own transfer. In an effort to avoid confusion, *Mob*⁺ will be used exclusively in this thesis to describe non-conjugative plasmids which are donated. The *trans* acting functions involved in mobilization are referred to as *mob* functions. The best-studied non-conjugative plasmids which can be mobilized by donation are ColE1, CloDF13 and RSF1010. Mobilization by conduction on the other hand, requires the formation of a co-integrate between the non-conjugative and the conjugative plasmids which is then transferred as a single unit. Conduction does not require the non-conjugative plasmid to encode any special functions and virtually all non-conjugative plasmids can be mobilized by this method.

In addition to functions involved in conjugal DNA transfer, many of the conjugative and non-conjugative plasmids isolated to date also encode genes which confer resistance to one or more antibiotic on their bacterial host. It was the concern over the spread of antibiotic resistance which initiated the study of diverse bacterial plasmids. Today the convenience of conjugation as a means of transferring DNA from cell to cell has encouraged detailed study of bacterial conjugation, especially among the less studied conjugal systems. One such system is the N transfer system of the conjugative plasmid pCU1. Preliminary studies have divided the *tra* genes of this plasmid into two groups the first of which has been shown to specify the formation and function of pili required for conjugal DNA transfer (Thatte and Iyer, 1983, Thatte *et al.*, 1985₁). The second group of pCU1 *tra* genes are not required for the formation of sex pili and are therefore believed to specify conjugal DNA metabolism functions, including the *cis* acting function designated as the *oriT*. Particular interest in the complex interactions between the *oriT* and the other *tra* functions involved in conjugal DNA metabolism has developed. The purpose of this

thesis is to expand the available knowledge of conjugal DNA metabolism in the N transfer system through a preliminary analysis of the *oriT* region and experiments on the specificity between *oriT* and *mob* functions.

2. LITERATURE REVIEW

2.1. Components of conjugal DNA metabolism

2.1.1. *oriT*

a) General structure and function

The origin of transfer has been described as the region required in *cis* for conjugal DNA transfer. Mutations within the *oriT* region which affect transfer cannot be complemented in *trans* by sequences carried on a separate DNA element within the same cell. In order for a DNA element to be transferred during conjugation, it must either carry its own functional *oriT* or be physically joined to a second element which carries a functional *oriT*.

The *oriTs* of conjugative plasmids F (Thompson and Achtman, 1979, Johnson, *et al.*, 1981), RK2 (Guiney and Yakobson, 1983), R1 (Ostermann *et al.*, 1984), R46 (Coupland *et al.*, 1987) and non-conjugative plasmids CloDF13 (Snijders *et al.*, 1983), ColE1 (Warren *et al.*, 1979) and RSF1010 (Derbyshire *et al.*, 1987) have been localized to specific restriction fragments and subsequently subcloned onto *Mob*⁻ plasmids. The resultant recombinant plasmids have been shown to be donated efficiently by the parent conjugative plasmid or in the case of ColE1, CloDF13 and RSF1010, the parental plasmid and an appropriate conjugative plasmid. The DNA fragments shown to carry the *oriTs* of the different plasmids have been relatively small, and in many cases have been only several hundred base pairs in size. For example, one of the smallest isolated *oriT* regions is that of RK2 which was found to

be contained within a 112 bp sequence (Guiney and Yakobson, 1983).

Although *oriT* has been described as the site at which conjugal transfer originates, implying a particular nucleotide, a more appropriate functional description is that it is a small sequence or region of DNA. Within this region, it is then useful to differentiate between at least two *cis* acting functions or domains: *nic* (site of strand nicking) and *bom* (basis of mobility). Because these two domains are so closely related, both physically and functionally, they are commonly referred to together as the *oriT*. *nic* is the actual site at which one strand of DNA is nicked while *bom* is the region extending on one or both sides of *nic* which acts as a recognition sequence for the nicking proteins (Warren *et al.*, 1978). This suggests that *nic* may lie within *bom*.

Evidence to support the belief that *oriT* should be considered a region rather than a specific site, is provided by studies on *cis* dominant transfer mutants created by transposon (Tn) insertions in the IncN plasmid pKM101 (Coupland *et al.*, 1985). In one instance, insertion of the 8.9 Kb transposon Tn1725, resulted in a 10,000 fold decrease in transfer frequency. Upon deletion of all but 60 bps of the transposon, transfer frequency was completely restored. These results suggest that *oriT* consists of a sequence of DNA more than the two base pairs comprising *nic* in which the components function only in close proximity to each other.

In addition to *nic* and *bom*, the *oriT* region must also encode functions which allow for the initiation of a complementary strand DNA synthesis within the donor and recipient (DCDS and RCDS). This may represent a third *cis* acting domain within the *oriT* region. As hypothesized with *nic* and *bom*, DCDS and RCDS functions may overlap with one or both *nic* and *bom*.

b) DNA sequence within the *oriT* region

The nucleotide sequence of the *oriT* regions of conjugative plasmids *F* (Thompson *et al.*, 1984), R100 (McIntire and Dempsey, 1987), R1 (Osterman *et al.*, 1984), R64 (Komano *et al.*, 1988), RK2 (Guiney and Yakobson, 1983) and R46 (Coupland *et al.*, 1987) and non-conjugative plasmids ColE1 (Bastia, 1978), CloDF13 (Nijkamp *et al.*, 1986), RSF1010 (Derbyshire *et al.*, 1987) and pSC101 (Willetts and Wilkins, 1984) have been examined. Comparisons between these sequences have been made in an effort to identify regions of homology which might represent *oriT* specific consensus sequences. Among the plasmids studied to date, no such general consensus sequence has been identified. This is not totally unexpected considering the general lack of interaction between the different *tra* systems and the variation in mobilization frequencies of non-conjugative plasmids by different conjugative plasmids. It should be noted however, that sequence homology has been observed between the *oriT* sequences of the closely related IncFI plasmid *F* and IncFII plasmids R100, R1 and ColB4. These plasmids all encode similar *F* type *tra* systems despite belonging to different incompatibility groups. Limited sequence homology has also been identified between *oriTs* of ColE1, CloDF13 and of the *F* plasmid which is able to mobilize them.

Despite the lack of a common consensus sequence within the *oriT* regions examined to date, investigators have identified some general features which are common to at least some of the sequences. Most of these features have been functionally associated with either the nicking of the DNA or the synthesis of the complementary strands of DNA within the donor and recipient cells.

Within the *oriT* of the IncF plasmids there is a noticeably AT-rich region of DNA. In a stretch of 86 base pairs, 69-72 (80-84%) are adenine or thymine residues. This feature has been seen in the replicative origins of many plasmids and is believed to promote melting of the complementary strands to assist in the opening of the double-stranded DNA during the initiation of replication (Kues and Stahl, 1989). The advantage of such a sequence in the immediate vicinity of the site at which the DNA breaks open and unwinding is initiated is obvious. Within the *oriT* regions of the other plasmids studied, the AT-rich stretches are significantly shorter (CloDF13, pSC101) or missing altogether (RK2, ColE1, RSF1010).

One structural feature which is found in all *oriT* regions studied to date is the inverted repeat. These inverted sequences range from 6 to 20 bps in length and number from one (pSC101) to four pairs (F, ColE1, CloDF13). In several instances the inverted repeats overlap (F, RSF1010), or represent regions of two fold rotational symmetry (pSC101, CloDF13, ColE1). In three cases where the exact location of *nic* has been determined (F, ColE1 and pSC101), the site is located within one of the inverted repeats, however this is not the case with the IncP plasmids RP4 (unpublished data reported by Ziegelin *et al.*, 1989) or RK2 (Guiney *et al.*, 1988).

There are two theories on the role of the inverted repeat within the *oriT* region. The first is that the secondary structure resulting from the hairpin loop or cruciform structure formed by the folding back of each strand onto itself, acts as a recognition feature to which the *mob* proteins and possibly host proteins can attach or bind. Ziegelin *et al.* (1989) demonstrated that the *traJ* protein of RP4 binds to a 19 bp inverted repeat sequence within the *oriT* region. The second proposal is that the hairpin loop may be involved in the synthesis of the complementary strand of

DNA within the recipient cell. This type of replication is found in the single-stranded bacteriophage G4. Primase binds to the double-stranded hairpin loop using it as a start for the transcription of a RNA molecule which is then used as a primer for DNA replication by host-encoded DNA polymerase (Sims *et al.*, 1980).

Another sequence believed to be involved in complementary strand synthesis is the *n'* recognition sequence AAGCGG. In this system, the *n'* protein (which is associated with several other host encoded proteins) binds to the recognition sequence followed by a primase enzyme forming a complex called the primosome complex. The complex travels along the DNA molecule until it encounters a promoter sequence (typified by the -10 TATA Pribnow box and -35 sequence) from which the transcription of a RNA primer molecule is initiated. This type of DNA synthesis priming mechanism is used by the single stranded phage ϕ X174 and has also been associated with the vegetative replication of narrow host range plasmids (Kues and Stahl, 1989). Both F and ColE1 possess *n'* recognition sites within their *oriT* sequences. In each case, the site is found within an inverted repeat and upstream from a -10 TATA sequence.

Less prevalent than the inverted repeat is the direct repeat. Of the *oriT* sequences known, only the IncP plasmid RK2 and the IncFII plasmid ColB4 contain direct repeats. In the case of RK2, a 12 bp sequence is repeated twice while in ColB4, an 8 bp sequence is repeated ten times. There is no homology between the repeats in the two plasmids. Direct repeats have been observed in the vegetative replication origin of wide host range plasmids (Kues and Stahl, 1989) including that of pCU1 (Krishnan and Iyer, 1990).

A final feature previously considered unrelated to conjugal transfer, but which

is now identified more frequently in *oriT* regions is the Integrative Host Factor (IHF) recognition sequence. IHF is a heterodimeric host-encoded protein required for the efficient integration of lambda bacteriophage into the *E. coli* chromosome. Although the phage encoded *int* gene product has been demonstrated to possess the topoisomerase activity required to break and reseal the DNA strands during the integrative recombination process, this activity is greatly reduced in the absence of IHF (Nash and Robertson, 1981). Nash and Robertson (1981) also demonstrated that while IHF can be shown to bind to the DNA it has no endonuclease or topoisomerase activity. The role played by IHF in the integration of lambda is believed to be one of enhancing the binding of the *int* gene product at the *attP* (attachment of phage) sites. Craig and Nash (1984) later identified three IHF binding sites within the *attP* region having the common sequence of T.PyAA...PuTTG(A/T)T. Additional studies on closely related phage ϕ 80 and P22 have refined the consensus sequence to PyAANNNTTGAT_(A/T) (Leong et al., 1985). Recently, an IHF binding site was identified in the conjugative plasmid R100 *oriT* region (McIntire and Dempsey, 1987) and R100 was shown to transfer at a reduced (100-450X) frequency from a IHF⁻ bacterial strain (Dempsey, 1987). Preliminary studies on this phenomenon has indicated that the reduced transfer frequency is accompanied by male-specific phage resistance suggesting that IHF affects the expression of the entire *tra* system (Dempsey and Fee, 1990). Other instances where IHF can be demonstrated to affect gene expression are the *E. coli* genes involved in the utilization of xylose (Friedman et al., 1984), the translation of the *cil* gene of lambda (Hoyt et al., 1982) and the function of the early operon of phage Mu (Goosen and van de Putte, 1984). The IHF has also been demonstrated to be necessary for the

maintenance of pSC101 (Gamas *et al.*, 1986) and IHF binding sequences have been observed within the replicative origins of several plasmids as well as several bacterial chromosomes (McIntire and Dempsey, 1987) suggesting that it may play a role in DNA replication. In the vegetative replication origin of pSC101, the IHF binding sites are located in the AT-rich region and are believed to enhance natural bending of the DNA in this region to allow closer association of essential DNA sites (Kues and Stahl, 1989). IHF binding within the F plasmid *oriT* region is also believed to promote bending of the DNA (Tsai *et al.*, 1990).

2.1.2. Relaxation complex

a) Relaxation complex of ColE1

Relaxation of supercoiled plasmid DNA was first demonstrated *in vitro* with the Mob⁺ plasmid ColE1 (Clewell and Helinski, 1969). ColE1 DNA isolated from *E. coli* cells by gentle lysis with lysozyme and non-ionic detergent and concentrated on a sucrose gradient was shown to be associated with a protein component. Upon treatment with ionic detergent sodium dodecyl sulfate (SDS), ethidium bromide, protease or heat (60°C), the DNA relaxed from the faster-sedimenting supercoiled form to the slower-sedimenting open circular or relaxed form. The supercoiled DNA-protein complex was subsequently referred to as a 'relaxation complex'.

SDS relaxed ColE1 DNA run on an alkaline sucrose gradient produced two bands of single stranded DNA, one linear and one circular. Preferential hybridization of one to the other rather than each to itself demonstrated that relaxation resulted from a strand-specific nick (Clewell and Helinski, 1970).

Further experiments with relaxed ColE1 DNA cut at a unique restriction site, produced three distinct bands on an alkaline agarose electrophoresis gel. One band corresponded in size to linear single strands of ColE1 DNA while the other two were smaller in size. The appearance of these latter two less-than-unit length fragments was taken as evidence that the *in vitro* relaxation event was not only strand specific but also site specific (Lovett *et al.*, 1974).

Studies on the protein component of the ColE1 relaxation complex have shown that there are three major proteins associated with the supercoiled DNA with molecular weights of 60,000, 16,000 and 10,000 daltons. The 60,000 molecular weight protein remains covalently bound to the 5' end of the nicked strand of DNA following relaxation (Blair and Helinski, 1975, Lovett and Helinski, 1975, Guiney and Helinski, 1975).

Correlation of the *in vitro* relaxation event observed with ColE1 and the mobilization of ColE1 was provided by Inselburg (1977). TrA insertion mutations which affected the relaxation event were also shown to result in a loss or decrease in mobilization frequency. The proteins involved in relaxation can therefore be considered to be involved in *mob* functions.

Two models have been proposed to explain the relaxation of the DNA-protein complex in the presence of ionic detergents, proteases and heat (Clewell and Helinski, 1970). In the first model, the DNA of the complex exists as a nicked molecule; however, supercoiling is maintained by the presence of the protein component which acts as a bridge over the single-stranded nick. The various treatments which cause relaxation (SDS, protease, heat and ethidium bromide) are seen to remove or denature the protein component, allowing the DNA to relax. In

the second model, the DNA is not nicked until the introduction of the relaxation conditions which are believed to trigger nicking of the DNA by the protein component. The basis of this model is the observation that some plasmids relax in the presence of SDS but not upon heat or pronase treatment. Furthermore, heat or pronase pretreatment prevents subsequent relaxation with SDS suggesting that a functional protein component is required for relaxation.

Relaxation experiments with ColE1 support the first model. ColE1 DNA relaxes under conditions which favour the denaturation or destruction of the protein component such as pronase or heat, as well as the presence of SDS and ethidium bromide.

b) Relaxation in F like plasmids

Like ColE1, the F plasmid has been isolated in the form of a supercoiled DNA-protein complex which demonstrates relaxation upon treatment with SDS, pronase and chymotrypsin. Incubation at 60°C resulted in a lower degree of relaxation and a subsequent loss of relaxation response to SDS or pronase (Kline and Helinski, 1971). This would suggest that the F relaxation complex fits the second model proposed by Clewell and Helinski, (1970) where the nicking protein may have been denatured by the heat treatment eliminating the appearance of activity upon subsequent activation by SDS or protease treatment. If this is so, the relaxation protein(s) must be protease-resistant.

Thompson *et al.*, (1989) visually demonstrated the relaxation of derivatives carrying the *oriT* of F using gel electrophoresis. Cleared lysates were prepared from strains carrying either an *oriT* derivative alone or in combination with a Tra⁺ F

derivative. After treatment with SDS, visualization on an agarose gel showed a band corresponding to the supercoiled form of the *oriT* derivative in both preparations while only the cleared lysate from the strain carrying both the *oriT* derivative and the conjugative plasmid produced a band migrating slightly slower than the relaxed form of the *oriT* derivative (CsCl isolated supercoiled DNA treated with topoisomerase). Treatment of the preparations with proteinase K prior to gel electrophoresis caused the slower migrating band to co-migrate with the relaxed DNA suggesting that a protein component remains associated with the *in vitro* relaxed DNA. They went on to demonstrate that the relaxation observed by the electrophoresis could be correlated to the ability of the plasmids to undergo conjugal transfer.

Everett and Willetts (1980) devised a novel *in vivo* relaxation assay involving lambda transducing phage carrying the F *oriT*. Denatured DNA from recombinant lambda phage packaged in cells carrying a functional F *tra* system demonstrated additional bands upon gel electrophoresis indicating the presence of a strand specific nick. This banding pattern could only be demonstrated with the strand known to contain *nic*. Further evidence was presented to indicate that this *in vivo* nicking required functional *traY* and *traZ* genes. From these experiments, it has been proposed that the TraY and TraZ proteins work together as an endonuclease.

c) Relaxation in other plasmids

In addition to ColE1 and F, relaxation in the presence of SDS has been demonstrated for plasmids R64 (IncI α), R28K (Inc unknown), R6K (IncX)(Kupersztoch-Portnoy *et al.*, 1974₁, Kupersztoch-Portnoy *et al.*, 1974₂) and RK2 (IncP)(Guiney and Helinski, 1979). Within this group, the R64 relaxation complex behaves like ColE1

showing relaxation in the presence SDS, pronase, ethidium bromide and heat while R28K and R6K, like F, do not relax with heat treatment and subsequent attempts to induce relaxation of heat-treated complexed DNA are unsuccessful. Unlike R28K and F, R6K relaxation is also not promoted by pronase treatment suggesting that if a functional protein is required for relaxation, this protein, unlike those found in F and R28K is pronase-sensitive.

2.1.3. Other functions involved in conjugal DNA metabolism

In addition to the *traYZ* nicking function, the F plasmid has been shown to encode three other *tra* genes which are believed to be involved in conjugal DNA metabolism. Abdel-Monem *et al.*, (1983) determined that the *traI* product was a DNA helicase I. It is believed that this protein facilitates the unwinding of the nicked DNA strand prior to transfer. DNA binding assays conducted by Lahue and Matson (1990) demonstrated that while the TraY protein binds to the DNA at the *oriT*, helicase I does not. There is evidence to support the theory that the TraZ (also called *traI'*) protein is the product of a second translational unit arising from within the terminal sequence of the *traI* transcript (Traxler and Minkley, 1987).

Two other F *tra* genes involved in conjugal DNA metabolism are *traM* and *traD*. Both functions are required for transfer but neither are involved in pili production or nicking. The TraM protein has been proposed as the signal which 'activates' the unwinding of the nicked strand (Everett and Willetts, 1980) while it has been suggested that the membrane bound, DNA binding TraD protein may act as a non-specific pore through which the single stranded DNA passes from the donor to

the recipient (Panicker and Minkley, 1985).

The IncP plasmid RP4 has been demonstrated to encode a primase enzyme which has been implicated in its wide host range (Lanka and Barth, 1981). Although mutations within the primase gene did not reduce transfer frequency between *E. coli* donor and recipient, they did reduce transfer from *E. coli* donors to *Proteus mirabilis* and *Salmonella* recipients suggesting that the *E. coli* encoded primase (*dnaG* product) could complement the mutations while the primase of other species could not. This primase activity has been shown to initiate synthesis of oligoribonucleotides on a defective M13 template *in vitro* (Yakobson *et al.*, 1990). Several IncI plasmids have also been shown to produce a primase enzyme. In the case where the *tra* systems were normally repressed, derepression resulted in increases in primase activity (Lanka and Barth, 1981).

2.2. Mobilization

2.2.1. Mobilization by donation

a) General features of donation

As previously indicated, mobilization by donation does not require physical interaction between the conjugative and non-conjugative plasmids. Mobilization by donation requires that the non-conjugative plasmid encodes its own *oriT* from which its transfer can be initiated. Each of the small non-conjugative, plasmids ColE1, CloDF13 and RSF1010 have been shown to encode a *cis* acting region required for mobilization (Warren and Clark, 1979, Van de Pol *et al.*, 1978; Derbyshire and

Willetts, 1987). In addition to their own *oriT* region each encodes some *trans* acting mobilization (*mob*) functions. Because these plasmids do not encode pili functions, the *mob* functions are assumed to be involved in conjugal DNA metabolism. In the case of ColE1, *mob* functions have been shown to include the relaxation complex proteins (Inselburg, 1977). Stable mating pairs are formed by the pili *tra* functions of the conjugative plasmid and transfer is mediated from the *oriT* of the mobilizable plasmid using its own *trans* acting *mob* functions and possibly some of the conjugal DNA metabolism *tra* functions of the conjugative plasmid.

Because the conjugative and non-conjugative plasmids move as separate elements either one or both can be transferred to the recipient cell during conjugation. As a result, the isolation of transconjugants containing the non-conjugative plasmid without the conjugative plasmid can be taken as evidence of donation (Warren and Clark, 1979).

A high frequency of transfer for the non-conjugative plasmid relative to the conjugative helper plasmid is also characteristic of donation and in many instances, the non-conjugative plasmid is transferred at a frequency comparable to the conjugative plasmid (Clark and Warren, 1979).

b) Interaction between the *mob* functions of non-conjugative plasmids and the *tra* functions of conjugative plasmids

Naturally occurring non-conjugative, *Mob*⁺ plasmids are mobilized with varying efficiency depending on the type of conjugative plasmid acting as the helper (Willetts, 1981). The information presented below provides evidence to suggest that while the conjugative functions involved in pili formation limit the interaction of the conjugative

plasmid with some non-conjugative plasmids the highest degree of specificity lies with the functions involved in conjugal DNA metabolism.

Mobilization of the IncQ, non-conjugative plasmid RSF1010 was first demonstrated with the IncI conjugative plasmid Collb (Guerry *et al.*, 1974). Subsequent data published by Willetts and Crowther, (1981) indicated that RSF1010 could be mobilized with differing efficiency by a variety of conjugative plasmids. They found that a second IncI plasmid, R64-11 mobilized RSF1010 as efficiently as Collb, as did several IncX plasmids (TP231, RTEM and RTEM^b). However the most efficient were two IncP plasmids, RP1 and R751, giving a mobilization frequency essentially equal to the transfer frequency of the conjugative plasmid. Among the less-effective conjugative plasmids, the IncW plasmids, R7K and R388 resulted in a mobilization frequency 1000 fold less than that of the IncI and IncX plasmids, while the three IncF and two IncN conjugative plasmids tested produced essentially no mobilization at all. These results suggest that the mobilization system of RSF1010 utilizes the P, I and X type transfer systems more efficiently than the F, N or W type transfer systems. As RSF1010 encodes some functions necessary for its own conjugal DNA metabolism, it is not clear whether the lack of mobility is due to an incompatibility of the RSF1010 plasmid with the pili produced by the conjugative helper plasmid, or if RSF1010 utilizes some of the conjugative plasmid's conjugal DNA metabolism functions which are not compatible with its *oriT* region. However, considering the wide range of pili produced by different plasmids, it seems more likely that differences in specificity between *oriT* and conjugative DNA metabolism functions of the helper plasmids are involved.

Similar experiments conducted on ColE1 demonstrated requirements for

specific *tra* functions by the mobilizable plasmid (Warren *et al.*, 1979). ColE1 was shown to be mobilized efficiently by F (IncF), R64-11 (IncI), R751 (IncP) and R388 (IncW). However, derivatives of ColE1 carrying deletions within its *mob* region demonstrated distinctly different mobilization frequencies depending on which of the four transfer systems acted as the helper conjugative plasmid. The results demonstrated that some of the ColE1 *mob* functions could not be complemented by any of the four conjugative plasmids while some could be complemented by the IncF and IncI transfer systems but not the IncW or IncP.

Van de Pol *et al.*, (1978) conducted a series of experiments on the mobilization of CloDF13 by transfer-deficient mutants of the F plasmid which further emphasizes the plasmid specificity of conjugal DNA metabolism functions. They demonstrated that CloDF13 was mobilized efficiently by not only F, but also by derivatives of F carrying mutations in *traI*, *traD* and *traM*. Tests conducted by Willetts (1980) confirmed these results for CloDF13 and found that ColE1 mobilization was also not affected by mutations in F *traM* or *traI*. Willetts also demonstrated that mutations in *traZ* did not affect the mobilization of either plasmid. These results suggested that either the *traI*, *traD*, *traM* and *traZ* gene products are not required for transfer of CloDF13 and ColE1 or that these non-conjugative plasmids encode similar functions specific for the non-conjugative plasmid. Plasmid specificity is supported by the fact that they do not complement the respective *tra* deficiencies in F. Since these four F *tra* genes are known to encode conjugative DNA metabolism functions, one can conclude that the *mob* functions of ColE1 and CloDF13 are involved in similar activities and that they are plasmid specific. Additional mutations in the F *traG* or *traN* resulted in loss of the ability to mobilize either CloDF13 or ColE1. Both

the F *traG* and *traN* genes have been shown to be involved in the stabilization of mating pairs (Ippen-Ihler and Minkley, 1986) which supports the view that non-conjugative plasmids do not encode *tra* functions involved in stable mating pair formation.

c) Interaction between the *mob* functions of different conjugative plasmids encoding similar transfer systems

Plasmid specificity for conjugal DNA metabolism functions can also be seen among the different plasmids encoding similar transfer systems (as defined by DNA homology, serology on pili and bacteriophage sensitivity). Willetts and Maule (1995) subcloned the *oriT* from F (IncFI), R1 (IncFII) and R100 (IncFII) and tested them for mobilization by the three parent conjugative plasmids and nine other conjugative plasmids belonging to either IncFI, IncFII or IncFIV. The results indicated that the transfer system of the parental plasmid recognized only its respective *oriT*, suggesting that each encodes a distinctive *oriT*. Among the nine other conjugative plasmids tested, two other IncFI plasmids mobilized the *oriT* of F as did one IncFII and two IncFIV plasmids; two IncFII plasmids mobilized the R100 *oriT*, one IncFII mobilized the R1 *oriT* and one IncFII plasmid (ColB4) did not mobilize any of the three chimeric *oriT* constructs. From these results, they concluded that there were at least four different *oriT* types or subgroups (F like, R1 like, R100 like and ColB4 like) within the F type transfer system. Complementation experiments between transfer deficient F *traM* mutants and the same group of F-type conjugative plasmids demonstrated the same distribution into the four subgroups which supports the belief that the *traM* gene product binds to DNA in the *oriT* region. Finlay *et al.*, (1986)

demonstrated that the *traY* product is not interchangeable between R100, R1 and F while the *traZ* product is. This information, combined with the knowledge that both *traZ* and *traY* products are required for *in vivo* nicking has led to the speculation that *traY* encodes for the more specific recognition protein(s) while *traZ* encodes for a less specific endonuclease protein(s).

Plasmids belonging to the IncP group have also been divided into subgroups based on their ability to mobilize small chimeric plasmids carrying the *oriT* of RK2 (Yakobson and Guiney, 1983). Chimeric plasmids carrying a 112 bp or 760 bp fragment containing only the *oriT* of RK2 and a larger construct carrying a 12 Kb region of RK2 including both the *oriT* and the region which encodes for the relaxation complex of RK2 were tested for mobilization by five IncP conjugative plasmids. The larger construct was efficiently mobilized by all five IncP plasmids while the smaller two constructs were mobilized 100-1000 times less frequently by two of the five conjugative plasmids. DNA hybridization experiments demonstrated that the two IncP plasmids unable to mobilize the small *oriT* constructs produced a different and much weaker hybridization pattern to a RK2 *oriT* probe than the three conjugative plasmids able to mobilize all three chimeric constructs. Apparently the difference in DNA sequence seen in the lack of homology within the *oriT* region resulted in the inability of the relaxation complex of the two non-homologous conjugative plasmids to interact with the *oriT* of RK2.

2.2.2. Mobilization by conduction

a) General features of conduction

The second method by which non-conjugative plasmids can be mobilized is conduction. In conduction the non-conjugative plasmid forms a co-integrate structure with the conjugative plasmid and transfer of the co-integrate originates from the *oriT* of the conjugative plasmid (Clark and Warren, 1979). Once in the recipient the two plasmids may or may not disassociate into separate elements.

Formation of the co-integrate structure in the donor cell can result from either *recA*-dependent recombination between sequences with extensive homology present on the two plasmids or by *recA* independent, site specific recombination. Due to the low frequency with which these events occur naturally, the frequency of transfer of the non-conjugative plasmid is very low as compared to that of the conjugative plasmid (Clark and Warren, 1979).

Because the non-conjugative plasmid can only be transferred if it is physically joined to the conjugative plasmid, transconjugants receiving the non-conjugative plasmid always receive the conjugative plasmid.

b) Conduction involving homologous recombination

The first demonstration of conjugal transfer by F is actually an example of conduction mediated by co-integration of F into the *E. coli* chromosome via homologous recombination between the IS (insertion sequence) elements carried by each (Davidson *et al.*, 1975). Because this type of recombination between homologous sequences is dependent upon the *recA* gene of the bacterial host,

conduction due to such interactions does not occur from *RecA⁻ E. coli* strains.

c) Conduction involving site-specific recombination

Site-specific recombination can occur by two mechanisms. In conservative site-specific recombination (CSSR), recombination occurs between two regions of sequence identity wherein strand breakage and joining occurs. The resultant recombination is reciprocal and no DNA is lost or synthesized. Both *oriV* and *oriT* sites have been demonstrated to be involved in CSSR-promoted conduction.

Conduction involving co-integration via CSSR at *oriT* has been demonstrated for both ColE1 and F plasmids. Warren and Clark (1980) demonstrated that upon transfer of ColE1 and chimeric plasmids carrying the *oriT* of ColE1 from the same *RecA⁻* cell, plasmid DNA isolated from transconjugants demonstrated co-integrate structures with a size the sum of ColE1 and the chimeric plasmid. Restriction analysis indicated that the joint between the two plasmids was within the 379 bp *oriT* region of the chimeric plasmid cloned from CciE1.

Studies conducted with F' constructs carrying deletions in *tra* have shown a site-specific recombination creating a junction within the region known to encode *oriT* (Horowitz and Deonier, 1985). The recombination sequence at the recombination site was the same in eight separate isolates, within the same inverted repeat sequence where *nic* sites had been previously identified (Thompson *et al.*, 1984).

Similar site-specific-promoted conduction has been observed with pSC101 chimeric plasmids carrying the *oriV* of the F plasmid. Kilbane and Malamy (1980) found that the presence of the F *oriV* on the chimeric plasmid resulted in a

mobilization frequency approximately 1000 fold less than the conjugative F plasmid from both a RecA^- and RecA^+ donor cell. Additional studies between pBR322 and pOX38 (Tra^+ F derivative carrying no known transposable elements) produced transconjugants carrying co-integrate structures with junctions lying within the fragment carrying the F *oriV* (O'Connor and Malamy, 1984). Sequencing of the junction showed no loss or synthesis of DNA.

Site-specific recombination resulting in the co-integration of a conjugative and non-conjugative plasmid can also occur during the transposition of a transposable element from one to another. This process does not involve homologous sequences. The transposition of the transposable element from one plasmid to the other results in non-reciprocal exchange of DNA involving DNA synthesis. Transposition mediated conjugation has frequently been observed to occur with the Tn3 system (Clark and Warren, 1979).

2.3. The N transfer system

pCU1 is a 39 Kb conjugative plasmid belonging to the IncN incompatibility group and encoding an N type transfer system. It encodes resistance to the antibiotics spectinomycin, streptomycin and ampicillin. It is considered to possess a wide host range having been isolated from *Aeromonas* spp., *Enterobacter* spp., *E. coli*, *Klebsiella aerogenes*, *Proteus* spp., *Providencia* spp., *Salmonella* spp., *Shigella* spp. and *Vibrio cholera* (Iyer, 1989) and transferred in the laboratory to *Caulobacter crescentus*, *Myxococcus*, *Pseudomonas*, and *Rhizobium* species where it is stably maintained.

The transfer system of pCU1 (Figure 4.1) has been characterized by Thatte et al., (1985). The region encoding the transfer functions of pCU1 is approximately 16 Kb in size, extending from 31.5 Kb on the pCU1 map through the 0 co-ordinate to approximately 8.2 Kb on the map. Transposon mutagenesis of this region has identified eight separate complementation regions, six of which are involved in pili production. The two complementation regions not required for pilus production are found clockwise of the *Sma*I site at approximately 2.7 Kb on the pCU1.

Unlike the longer, flexible pili of the F system, the N transfer systems encode short, rigid sex pili, more like those encoded by the P, W and M transfer systems (Bradley, 1979). Due to the nature of these pili, bacterial matings are found to be more efficient when carried out on solid rather than in liquid medium (Dennison and Baumberg, 1975, Bradley et al., 1980). pCU1 is susceptible to two male specific bacteriophage, a filamentous, single stranded, DNA phage, I_{ke} (Khatoon, et al., 1971) and an icosahedral, double stranded DNA phage PRD1.

DNA-DNA hybridization experiments have demonstrated sequence homology among several IncN plasmids. In several cases the DNA fragments hybridizing to the pCU1 derived probe, co-migrated with pCU1 fragments known to encode *tra* functions (Konarska-Kozłowska and Iyer, 1983). Most striking is the similarity in restriction digest pattern within the *tra* regions of pCU1 and the R46 Tra⁺ derivative pKM101 (Winans and Walker, 1985₂).

Other IncN plasmids currently being studied in other laboratories include N3 (Ando and Arai, 1981), R15 (Dobritsa et al., 1985) and R46 and its Tra⁺ derivative, pKM101 (Coupland et al., 1987); however, the *tra* system of R46 is the only other N type system which has been studied in any detail. In comparison to pCU1, the

transfer region of pKM101 is approximately 17 Kb in size and encodes 15 complementation regions, 11 of which are directly involved in conjugal transfer. Three additional complementation regions encode transfer related functions such as entry exclusion, fertility inhibition of IncP plasmids, and an endonuclease (Winans and Walker, 1985₂). The endonuclease has been shown to be unnecessary for transfer.

pMUR545 and pMUR274 are two additional plasmids which encode an N transfer system, as demonstrated by their susceptibility to lke bacteriophage. Isolated in the years preceding the extensive use of antibiotics, these two plasmids are cryptic in that they encode no selectable antibiotic marker (Datta and Hughes, 1983, Hughes and Datta, 1983). Recent work from this laboratory suggests that although these two pMUR plasmids encode N transfer systems, they may not belong to the N incompatibility group.

3. METHODS AND MATERIALS

3.1 Bacterial strains, bacteriophages and plasmids

See Tables 3.1, 3.2 and 3.3.

3.2 Growth and maintenance of bacterial cultures

Cultures were grown in either Difco Antibiotic medium #3 (Penassay broth) or L broth (1.0% tryptone, 0.5% NaCl, 0.5% yeast extract). Solid media contained 1.5% agar. Antibiotics were added from filter sterilized stock solutions to solid media in the following concentrations: chloramphenicol, Cm, 30 $\mu\text{g/ml}$; spectinomycin, Sp, 50 $\mu\text{g/ml}$; ampicillin, Ap, 30 $\mu\text{g/ml}$; kanamycin, Km, 50 $\mu\text{g/ml}$; tetracycline, Tc, 10 $\mu\text{g/ml}$; nalidixic acid, Nal, 50 $\mu\text{g/ml}$; rifampicin, Rif, 100 $\mu\text{g/ml}$. For Cm, Km, Ap and Sp, one half the concentration was used in liquid media. Tc was added to liquid media in the same concentration. When Ap was used to select for low frequency transfer of plasmids (requiring high concentrations of mating mixture to be plated) clavulanic acid was added to the medium at a concentration of 1.5 $\mu\text{g/ml}$ to inhibit the growth of feeder colonies around the Ap^r transconjugants. Neither Nal or Rif was used in liquid media. Antibiotic selection for plasmid markers was maintained at all times to ensure plasmid stability.

Unless otherwise stated, all cultures were grown at 37°C. All liquid cultures were grown in a reciprocal shaker.

Stock cultures were maintained at -20 or -80°C in broth containing 40% glycerol.

Table 3.1 *E. coli* strains used in this study

Strain	Genotype/Phenotype	Reference
HB101	<i>F⁻ hsdS20 recA13 ara-14 proA2 lacy1 galk2 rpsL20 xyl-5 mtl-1 λ⁻</i>	Bolivar, et al., 1977
C600Nal	<i>F⁻ thi-1 thr-1 leuB6 lacy1 tonA21 supE44 λ⁻ Nal^r</i>	this laboratory
JM101	<i>supE Δ (lac-proAB) (F[']traD36 proAB⁺lac^qlacZ M15)</i>	Messing, 1973
DH5αF [']	<i>endA1 hsdR17 supE44 thi-1 recA1 gyrA relA1 o80 dlacZ M15 Δ (lacZYA-argF)U169</i>	Liss, 1987

Table 3.2 Bacteriophages used in this study

Bacteriophage	Reference
M13mp18 & M13mp19	Messing and Vieira, 1982
M13K07	Vieira and Messing, 1987
lambda::Tn10	Kleckner, 1931

Table 3.3 Plasmids used in this study

Plasmid	Phenotype	Comments or pCU1 co-ordinates	Reference or Source
pCU1	Sp ^r Ap ^r Sm ^r Tra ⁺		Konarska-Kozłowska and Iyer, 1981
pCU29	Sp ^r Ap ^r Sm ^r Km ^r Tra ⁺	Tn5 at 9.55	Thatte <i>et al.</i> , 1983
pCU56	Cm ^r Tra ⁻ Mob ⁺ ^b	7-11.5	Thatte <i>et al.</i> , 1985 ₂
pCU57	Cm ^r Tra ⁻ Mob ⁺	0-9.55	•
pCU101	Cm ^r Tra ⁺	27.5-9.55	Thatte and Iyer, 1983
pCU109	Cm ^r Tra ⁺	27.5-11.2	•
pCU401	Km ^r	32.5-21	Thatte <i>et al.</i> , 1985 ₂
pCU404	Cm ^r Tra ⁻ Mob ⁺	7-11.5	•
pVT30	Cm ^r Km ^r Tra ⁺	Tn5 at 9.15 in pCU109	Thatte <i>et al.</i> , 1985 ₁
pMK2004	Ap ^r Km ^r Tc ^r Tra ⁻ Mob ⁻	N/A	Kahn <i>et al.</i> , 1979
pIBI76	Ap ^r Tra ⁻ Mob ⁻	N/A	Dente <i>et al.</i> , 1983
R46	Ap ^r Sp ^r Sm ^r Tc ^r Tra ⁺	N type <i>tra</i>	T. Kato
R113	Tc ^r Tra ⁺	N type <i>tra</i>	J. Jorgensen
R199	Tc ^r Tra ⁺	N type <i>tra</i>	N. Datta
R45	Ap ^r Tc ^r Tra ⁺	N type <i>tra</i>	N. Datta
pTM4245	Sm ^r Tc ^r Tra ⁺	N type <i>tra</i>	C. Monti-Bragadin
pMUR274	Tra ⁺	N type <i>tra</i>	Hughes and Datta, 1983
pSP274	Tc ^r Tra ⁺	pMUR275::Tn10	this study
pMUR545	Tra ⁺	N type <i>tra</i>	Hughes and Datta, 1983
pSP545	Tc ^r Tra ⁺	pMUR545::Tn10	this study
pKM101 <i>traH1143</i>	Ap ^r Km ^r Tra ⁻ Mob ⁺	N type <i>tra</i>	Winans and Walker, 1985 ₂
pKM101 <i>traI1136</i>	Ap ^r Km ^r Tra ⁻ Mob ⁺	N type <i>tra</i>	•
pKM101 <i>traJ35</i>	Ap ^r Km ^r Tra ⁻ Mob ⁺	N type <i>tra</i>	•
pKM101 <i>traK1217</i>	Ap ^r Km ^r Tra ⁻ Mob ⁺	N type <i>tra</i>	•
R100-1	Sp ^r Sm ^r Cm ^r Tc ^r Tra ⁺	F type <i>tra</i>	N. Datta
R386	Tc ^r Tra ⁺	F type <i>tra</i>	N. Datta
RA3	Sp ^r Sm ^r Cm ^r Tra ⁺	W type <i>tra</i>	E. Lederberg
S-a	Sp ^r Cm ^r Km ^r Sm ^r Tra ⁺	W type <i>tra</i>	N. Datta
R621a	Tc ^r Tra ⁺	I type <i>tra</i>	N. Datta
R64-11	Sm ^r Tc ^r Tra ⁺	I type <i>tra</i>	B. Wilkins
R446b	Sm ^r Tc ^r Tra ⁺	M type <i>tra</i>	N. Datta
pBRK4013	Ap ^r Tra ⁺	P type <i>tra</i>	this lab
pBGL	Ap ^r Km ^r Tra ⁻ Mob ⁺	3.3-7, 8.48-9.15	this study

Plasmid	Antibiotic marker	Nucleotide co-ordinates of pCU1 <i>oriT</i>	Reference or Source
pSP1	Ap Km	all (1-830)*	this study
pSP2	Ap Km	all	•
pSP3	Ap Km	all	•
pBB15	Ap Km	all	•
pSP11	Ap Km	1-551	•
pSP12	Km	400-692	•
pSP13	Ap	1-419	•
pSP14	Ap	1-554	•
pSP15	Ap Km	1-400, 554-692	•
pSP16	Ap	400-554	•
pSP17	Ap Km	1-419, 562-830	•
pSP18	Km	525-692	•
pSP19	Km	436-692	•
pSP20	Km	487-692	•
pSP21	Km	400-436, 487-692	•
pSP22	Km	1-487	•
pSP23	Km	1-436	•
pSP25	Ap Km	368-692	•
pBP1	Ap Km	1-692	•
pBP11	Ap Km	1-551	•
pBP50	Ap Km	all	•
pBS6	Ap Km	all	•
pBS8	Ap	all	•
pBS17	Ap	all	•
pBS18	Ap	all	•
pBS22	Ap	all	•
pBS32	Ap Km	all	•
pBS38	Ap Km	all	•
pBS47	Ap	1-419	•
pBE26	Ap Km	unknown	•
pBE42	Ap Km	562-830	•
pBE44	Km	all	•
pBE48	Km	all	•
pBE60	Km	all	•

* nucleotides 1-830 corresponds to approximately 9.15-8.32 Kb on the pCU1 map.

3.3 Bacterial matings

a) Large scale filter matings (for determining transfer frequencies)

Overnight cultures of donor cells were diluted 0.1 ml into 5 ml PB or LB with the appropriate antibiotics and grown for 3 hr (approximately 5×10^8 cells per ml). 1 ml of over night recipient culture and 1 ml of 3 hour donor culture were filtered together through a 47 mm, 0.45 μ m pore membrane filter. The filters were incubated on LBA or PA media without antibiotics for 2 hours at 37°C then washed off into 1 ml saline (0.85% NaCl). Dilutions were prepared in saline and 0.1 ml of selected dilutions were plated on PA media containing antibiotic selection for both the recipient and the plasmid to be transferred. Where donor cells contained more than one plasmid (ie mobilization experiments), selection for each plasmid marker was applied separately so that the transfer frequency of individual plasmids could be calculated. Plates were incubated at 37°C for 24 hours.

Transconjugant counts/ml of mating mixture were calculated as the number of colony forming units $\times 10 \times$ the dilution factor. Wherever possible, plates containing 30-300 colonies were used preferentially over those containing more than 300 or less than 30 colonies.

Viable counts of donor cultures were obtained by the procedure outlined above except that 2 ml of donor culture was filtered in place of 1 ml of each of donor and recipient. The diluted cultures were plated on LBA or PA containing the antibiotic(s) used to select for the plasmid(s) in the donor cell. Donor count per ml of culture was calculated as the number of colony forming units $\times 10 \times 0.5 \times$ the dilution factor.

To ensure that neither donor nor recipient could grow on the media used to select for transconjugants, 0.1 ml of the recipient culture or donor saline suspension was plated on each selective medium used to grow the transconjugants of the corresponding mating.

Transfer frequencies were determined by dividing the transconjugant count by the donor count to give the number of transconjugants receiving a particular marker per donor cell carrying that marker. In mobilization experiments, in order to compensate for the variation of transfer ability of the various helper plasmids, mobilization frequencies are given in terms of the number of transconjugants receiving the marker of the mobilizable plasmid per transconjugant receiving the marker of the conjugative plasmid.

b) Mini-filter matings (for construction of strains)

50 μ l of overnight recipient culture was mixed with 50 μ l of overnight or log phase donor culture on a 27 mm, 0.45 μ m pore size filter disc on an LBA or PA plate and incubated for 1-2 hours. The filter was rinsed in 1 ml saline and 0.1 ml of selected dilutions plated on LBA or PA media containing antibiotics to select for recipients receiving the conjugative or mobilizable plasmid. Plates were incubated for 24-48 hours. Controls were run on both the donor and recipient cultures. An isolated colony was streaked for purity on agar plates containing antibiotics to select for all plasmids.

c) Pellet matings (for screening isolates for transfer ability)

0.5 ml of overnight recipient culture was mixed with 0.5 ml of log phase donor culture in a sterile 1.5 ml Eppendorf tube. The mixture was centrifuged for 2-3 min and the supernatant discarded. The pellet was incubated for 2 hours at 37°C. After incubation the pellets were resuspended in 1 ml saline and dilutions plated on media selective for the recipient and the marker carried by the plasmid of interest.

Controls were run on both recipient and donor(s).

3.4. Replica plating for determining co-transfer of conjugative and non-conjugative plasmids

Colonies from plates selecting for the transfer of the non-conjugative plasmid (after large scale filter matings) were transferred by sterile toothpicks to plates containing antibiotics used to select for the conjugative plasmid marker. The ratio of transconjugants carrying only the non-conjugative plasmid without the conjugative helper plasmid is given as the number of isolates sensitive to the conjugative plasmid antibiotic marker per isolate resistant to the non-conjugative antibiotic marker.

3.5. Large scale plasmid DNA extraction by CsCl gradient

0.5 ml of overnight culture was spread onto each of 6 LBA or PA containing the appropriate antibiotic. The plates were incubated overnight at 37°C. The cells were washed from the plates with a total of 40 ml sucrose buffer (25% sucrose in

0.05 M Tris, pH 8.0) and transferred to two SS-34 centrifuge tubes. To each tube, 0.5 ml of lysozyme (10 mg/ml) and 3 ml 0.25 M EDTA was added. The mixture was left on ice for 5 min and 4 ml of Triton X-100 lytic solution (Triton X-100, 2 %, Tris, 50 mM, EDTA, 60 mM, pH 8.0) added. If necessary, cell lysis was promoted by incubation at 60°C for 5-10 min. Once lysis had occurred the solution was centrifuged at 15,000 RPM for 30-60 min at 4°C. The supernatant was collected and 7 ml of a 40% PEG 6000 solution in 2 M NaCl was added. Precipitation was allowed to proceed overnight at 4°C followed by centrifugation at 6,000 RPM for 5 min at 2°C. The two pellets were resuspended in a total of 3.2 ml TE buffer (Tris-HCl, 0.01 M, EDTA, 0.001 M, pH 8.0) to which 4.000 g CsCl and 0.5 ml ethidium bromide (10 mg/ml) was added. The DNA was centrifuged overnight at 60,000 RPM and the plasmid DNA band removed from the tube with a syringe. The ethidium bromide was extracted from the DNA with CsCl saturated isopropanol and the CsCl removed by dialysis in TE buffer. The DNA was precipitated with one fifth volume of 10 M ammonium acetate and two volumes of absolute ethanol at -20°C overnight followed by a 1 hour spin at 8,000 RPM in an HB4 rotor in the Sorval centrifuge. The DNA pellet was dried at 50°C under vacuum, resuspended in 25-100 μ l of dH₂O and stored at 4°C.

3.6. Rapid 'mini-prep' plasmid DNA extraction

The method used is a variation of the boiling method described by Holmes and Quigley (1981). A 5 ml overnight culture was pelleted in a bench top centrifuge and resuspended in 400 μ l STET buffer (sucrose, 8%, Triton-X-100, 5%, EDTA, 50

mM, Tris, 50 mM, pH 8.0). The suspension was transferred to 1.5 ml Eppendorf tube containing 20 μ l lysozyme (10 mg/ml) and placed in a boiling bath for 50 sec. The tube was then spun in the microcentrifuge for 15 min at 4°C and the gelatinous pellet removed with a sterile toothpick. The DNA was precipitated with one fifth volume 10 M ammonium acetate and two volumes ethanol on ice for 30 min followed by a 30 min spin at 4°C. The pellet was washed with 70% ethanol and dried at 50°C under vacuum. The dried pellet was resuspended in 20 μ l of dH₂O.

3.7. Restriction digest of DNA

Restriction endonuclease digests were carried out in 10 or 20 μ l reactions using TA restriction buffer (33 mM Tris acetate, pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol, 100 μ g/ml gelatine). For *Sall* digests, 1.5 M NaCl was added. Digests were incubated for 1-2 hours at 37°C except *SmaI* digests which were incubated at 24°C. Digests typically contained 1 μ l of CsCl purified plasmid DNA or 2 μ l of mini-prep DNA and 1 μ l of enzyme (4-12 units/ μ l). Restriction enzymes were from BioLabs, Boehringer-Manheim, Amersham or Pharmacia.

3.8 Agarose gel electrophoresis of restriction digests

Bio-rad horizontal, submerged mini-gel apparatus was used to analyze restriction digests. Bio-rad agarose (0.7-1.2% w/v) was dissolved in Tris-acetate electrophoresis buffer, TAE (80 mM Tris, 40 mM sodium acetate, 4 mM EDTA, pH 8.3) by heating in a microwave oven. The gels were left to set for 30-60 min.

For mini-prep digests, 2 μ l of stop solution (30% glycerol, 0.05% bromophenol blue, 5 mM EDTA) was added to a 10 μ l digest and loaded onto the gel; for CsCl DNA digests, 1-2 μ l of a 10 μ l digest was mixed with 5 μ l of stop solution and loaded onto the gel.

Gels were run at 60-100 volts for 1-3 hours or until the tracking dye was approximately one half inch from the end of the gel. Gels were stained in ethidium bromide solution (0.4 mg/ml) 5-10 min and destained in dH₂O for 10-60 min. Gels were photographed using a Polaroid Land camera and Polaroid type 57 or 55 film.

BRL 1 Kb DNA ladder was run on each gel as a DNA size standard. The size of the fragments, in base pairs (bp) were: 12216, 11198, 10180, 9162, 8144, 7126, 6108, 5090, 4072, 3054, 2036, 1636, 1018, 517/ 506. Fragment size of digested DNA were determined by computer program DNA Frag (Schaffer and Sederoff, 1981)

3.9. Vertical 'Eckhardt Gel' electrophoresis for screening plasmid populations

This method is a variation of the method described by Eckhardt (1978). 1% agarose gel was prepared in Tris Borate Buffer, TBE (89 mM Tris, 89 mM boric acid, 25 mM EDTA, pH 8.0-8.3). 1 ml of each overnight culture to be screened was pelleted in an Eppendorf tube, washed twice with TBE and resuspended in 100 μ l of cell suspension solution (20% w/v Ficoll 400 in TBE). Each gel well was loaded sequentially with 30 μ l lysis mixture (7500 units/ml lysozyme, 0.3 units /ml ribonuclease, 0.05 % bromophenol blue, 20% ficoll 400 in TBE), 15 μ l cell suspension, 60 μ l overlay A (0.2% SDS, 10% Ficoll 400, 2.5 mM EDTA in TBE) and 60 μ l overlay B (0.2% SDS, 5% ficoll 400 2.5 mM EDTA in TBE). Only the lysis

mixture and cell suspension layers were mixed. Gels were run at 20 volts for 1 hour followed by 40-65 volts for 16-20 hours depending on the size of plasmid(s) to be separated. Gels were stained and photographed as outlined above (Section 3.8).

3.10. Cloning restriction fragments into vector DNA

a) Shotgun cloning

1-2 μg of CsCl isolated insert and vector DNA were digested with the appropriate restriction enzymes in separate reactions. 1-5 μl of insert DNA digest was mixed with 1-3 μl of vector DNA digest such that the insert DNA was present in at least two fold excess with respect to the vector DNA. To this mixture 4 μl of 5x BRL ligation buffer (330 mM TRis-HCl, pH 7.6, 33 mM MgCl_2 , 50 mM DTT, 330 mM ATP) and dH_2O to bring the volume to 19 μl . To this 1 μl of BRL T4 DNA ligase (1 unit/ μl for cohesive ends, 20,000 units/ μl for blunt ends) and the reaction was incubated at 15°C overnight. 5-10 μl of the ligation reaction was transformed into the desired host (see Section 3.11).

b) Extraction of DNA restriction fragments from agarose gels by Gene clean procedure for subcloning

1-2 μg of CsCl isolated insert DNA was digested and run on a TAE electrophoresis gel. The gel was stained and viewed under long wave U.V. light and the desired fragment cut from the gel. The agarose fragment was weighed and 2-3 volumes 6 M sodium iodide solution was added. This was held at 50°C for 5 min. 5 μl of Gene Clean Glass Milk™ preparation was added and the preparation held on

ice for 5 min. The DNA coated glass milk was pelleted by spinning in a microfuge for 5 sec and the supernatant discarded. The pellet was washed in 300-400 μ l of NEW solution three times and the final pellet resuspended in 10 μ l of dH₂O and incubated at 50°C for 2-3 min. The glass milk matrix was pelleted 30 sec in the microfuge and the supernatant containing the DNA was retrieved. Up to 10 μ l of this insert DNA preparation was ligated to 1 μ l of vector DNA digestion, in a 20 μ l reaction as outlined above (Section 3.10a).

3.11. Bacterial transformation

An overnight culture of the strain to be transformed was diluted 0.5 ml in 30 ml LB and reincubated for 2.5-3 hours at 37°C. The cells were chilled on ice for 20 min then pelleted at 5000 RPM for 5 min at 4°C in an SS-34 rotor and Sorval centrifuge. The cells were resuspended in 7.5 ml of ice cold 100 mM MgCl₂ and repelleted. The cells were then resuspended in 1-1.5 ml of ice cold 100 mM CaCl₂ and held on ice for a minimum of 30 min. 0.2 ml aliquots of competent cells were mixed with DNA in chilled glass tubes and left on ice for an additional hour.

Generally, 1 μ l of CsCl DNA, 2-5 μ l of mini-prep DNA or 5-10 μ l of ligation mixtures diluted to 50 μ l in cold sterile dH₂O was used to transform cells. After one hour on ice, the transformed cells were heat pulsed at 37°C for 5 min then transferred to ice for 10 min. 1 ml of LB was added to each transformation and the cells were incubated at 37°C for 45-90 min with gentle shaking. The cells were plated on LBA or PA containing the antibiotic to select for the plasmid marker. Plates were incubated at 37°C overnight and isolated colonies streaked for purity on selective

plates.

3.12. Bal31 deletion mutagenesis

An excess (approximately 5 μ g) of CsCl purified plasmid DNA was digested to completion at a unique restriction site next to the region to be deleted in a 30 μ l reaction. A trial Bal31 digestion was carried out to determine the appropriate temperature and incubation time required to obtain the desired deletions. 5 μ l of the linearized DNA was added to 17 μ l dH₂O and 6 μ l 5 X Bal31 reaction buffer (300 mM CaCl₂, 300 mM MgCl₂, 1.5 M NaCl, 500 mM Tris-HCl, 25 mM EDTA, pH 8.0). The tube was prewarmed to the appropriate incubation temperature and 2 units of Bal31 nuclease added. 5 μ l aliquots were removed at specified time intervals and transferred to tubes containing 1 μ l of 0.5 mM EGTA to terminate the reaction. These samples were run on a TAE agarose gel to determine the extent of the deletion at the various incubation times. In general, for deletions less than 2 Kb in size, incubation was carried out at 24°C for 2-10 min, larger deletions were created at 30°C for 2-20 min.

The process was repeated with 5 additional reactions prepared as for the trial and incubation was carried out for 5 different time periods as dictated by the trial findings. Each reaction was terminated after the appropriate incubation period by the addition of 3 μ l 0.5 mM EGTA. The reactions were pooled, extracted with phenol, phenol and chloroform (1:1) and chloroform and then precipitated with ammonium acetate and ethanol. The dried pellet was resuspended in dH₂O.

The overhanging ends generated by the Bal31 nuclease were filled in a

reaction containing 0.125 mM dNTP and 2-3 units Klenow polymerase in Hin buffer (100 mM Tris-HCl pH 7.4, 100 mM MgCl₂, 500 mM NaCl) incubated at 30°C for 1 hour. The reaction was terminated by incubation at 70°C for 10 min.

*Bam*HI linkers were added to the flush ends in a reaction containing 1 unit T4 DNA ligase (BRL) and 50 µg/ml linkers (Amersham) in BRL ligation reaction buffer (see above) incubated overnight at 12-15°C. This ligation reaction was then digested with excess *Bam*HI enzyme (100 units) at 37°C for 2 hours in a 100 µl reaction. The digested DNA was precipitated with ammonium acetate and ethanol, resuspended in dH₂O and religated in a 20 µl reaction overnight. 5-10 µl of this ligation reaction was diluted to 50 µl with dH₂O and used to transform the desired bacterial host with selection for the antibiotic marker.

3.13. Insertion of Tn10 into the pMUR plasmids

Plasmids pMUR274 and pMUR545 do not encode for a selectable antibiotic marker. The small, non-conjugative plasmid R300b is Sm^r and mobilized by the pMUR plasmids. It is co-maintained in the bacterial cell to act as a marker for pMUR conjugal transfer. In order to eliminate the need for R300b, an antibiotic marker was inserted into the pMUR plasmids by transposition. 0.2 ml of 4 hr J53-2/pMUR274/R300b and J53-2/pMUR545/R300b cultures were each incubated at 30°C with 0.2 ml of a suspension containing 10⁸ lambda::Tn10 phages for 2 hours. The mixture was then mated with 0.1 ml overnight C600Nal culture by large filter mating as outlined above (Section 3.3). The transconjugants were selected on Nal+Tc plates and further screened for sensitivity to Sm by replica plating. One Sm^r Tc^r isolate was

streaked for purity on a Nal+Tc plate.

3.14. Single-stranded sequencing

a) Growth and identification of recombinant phage

pIBI76 recombinant DNA was transformed into DH5 α F' as outlined above (Section 3.11), however IPTG (0.5 mg/ml) and X-gal (40 μ g/ml) were added to the agar medium along with ampicillin (30 μ g/ml). White colonies were kept for further screening. 10 and 100 μ l of JM101 cells transformed with recombinant M13 ligation mixtures were mixed with 200 μ l of log phase DH5 α F' cells, 20 μ l IPTG solution (24 mg/ml) and 10 μ l X-gal (100 mg/ml) and 2.5 ml H top agarose (1.0% tryptone, 0.8% NaCl, 0.8% agarose) and plated on H bottom agar (1.0% tryptone, 0.8% NaCl, 1.2% agar) and incubated overnight at 37°C. White plaques were chosen for further screening.

b) Horizontal gel electrophoresis for screening M13 inserts

2.5 ml of 2X YT broth (1.6% tryptone, 1.0% yeast extract, 0.5% NaCl) were inoculated with individual white recombinant M13 plaques and 0.1 ml of overnight or log phase DH5 α F' cells or white recombinant pIBI76 transformants and 0.1 ml of M13K07 helper phage (10^7 PFU/ml) and grown overnight with vigorous shaking. One blue plaque or colony was also cultured as a control. 1 ml of each culture was pelleted and 20 μ l of the supernatant mixed with 2 μ l of 25% SDS in a microtitre plate well. Lysed phage suspension was run on a 0.7% TBE agarose gel at 100 mAmps for approximately 2 hours. The gels were stained and photographed as

outlined above (Section 3.8). The cell pellets (from above) of isolates showing reduced mobility with respect to the control blue plaque or colony isolate were subjected to mini-prep DNA isolation (Section 3.6) and restriction analysis (Section 3.7) to confirm insert.

c) Isolation of single stranded template DNA

1 ml of supernatant from the culture used for ss phage DNA screen by gel electrophoresis was precipitated with 116 μ l 10 M ammonium acetate and 155 μ l 40% PEG 1 hour on ice or overnight at 4°C. The DNA was pelleted at 4°C for 5 min in a microfuge and resuspended in 120 μ l TE, extracted with phenol, phenol and chloroform (1:1) and chloroform and precipitated with ammonium acetate and ethanol. The resulting pellet was washed with 70% ethanol, dried and resuspended in 30 μ l of dH₂O.

d) Sequencing reactions

Sanger, dideoxy sequencing procedure was carried out as outlined in the US Biochemical Sequenase product pamphlet. 7 μ l of single stranded DNA was mixed with 1 μ l (0.5 μ moles/ μ l) primer DNA and 2 μ l 5X sequencing buffer (200 mM Tris-HCl, pH 7.5, 100 mM MgCl₂, 250 mM NaCl) at 65°C for 5 min and allowed to cool slowly in the same beaker of water to 37°C. The universal primer was used to sequence M13 recombinant derivatives while the reverse sequencing primer was used to sequence the pIBI76 recombinant derivatives. To the annealed template was added: 1 μ l 0.1 M DTT, 2 μ l diluted labelling mix - either 1:4 or 1:9 dilution was used

(1.5 or 0.75 μ M dGTP, dCTP and dTTP) depending on whether the region of the DNA to be sequenced was close to the primer or further away, 1 μ l deoxyadenosine-³⁵S-5'-triphosphate (10 μ C) and 2 μ l of dilute Sequenase[™] enzyme. The tubes were incubated 5 min at room temperature after which time 3.2 μ l was transferred to each of 4 prewarmed tubes containing 2.5 μ l of one of the 4 termination mixtures (50 mM NaCl, 80 μ M dGTP, 80 μ M dATP, 80 μ M dCTP, 80 μ M dTTP, 8 μ M of either ddGTP, ddATP, ddCTP or ddTTP) and incubated 5 mins at 37°C. The reaction was terminated with the addition of 5 μ l STOP solution (95% formamide, 20 mM EDTA, 0.05% Bromophenol blue, 0.05% xylene cyanolFF).

e) Sequencing acrylamide gel electrophoresis

A 7.5% acrylamide gel containing 7 M urea in TBE buffer was used for sequencing electrophoresis. Sequencing apparatus was from BRL. The gel was prerun at 1000 volts for 45 min. Samples were boiled for 2-5 min and one half of each reaction (2.5 μ l) loaded. The gel was run at 1200-1500 volts, 30-45 mamps for 30-60 min after the bromophenol blue ran off the gel. The rest of the sample was denatured and loaded for the second run at 1500-1800 volts, 30 mamps until the bromophenol blue ran off the gel. The gel was fixed in a solution of 10% acetic acid and 10% methanol for 30 min and dried at 80°C under vacuum for 1 hour. X-Omat AR film was exposed for 24-48 hours at room temperature and developed in a film processor.

3.15. Relaxation assay

The procedure used to assay the relaxation of plasmid DNA was a modified version of the procedure first introduced by Clewell and Helinski, 1969.

a) Preparation of the cleared lysate

1 ml of an overnight culture of an HB101 carrying the plasmid grown in LB + antibiotic was pelleted in an microfuge tube. The cells were resuspended in 1 ml saline and 0.5 ml of this used to inoculate 30 ml of a 1 M Tris buffered medium (pH 7.3) containing: 0.2% NH_4Cl , 0.5% NaCl, 0.037% KCl, 0.0001% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.002% NaSO_4 , 0.5% casamino acids, 0.025% deoxyadenosine, 0.025% thiamine and 0.2% glycerol. 100 μCi of ^3H -thymine was added and the cells were incubated at 37°C over night with shaking.

The cells were pelleted in a bench top centrifuge and resuspended in 1 ml of sucrose buffer (Section 3.5). After 5 min on ice, 20 μl lysozyme (10 mg/ml) was added and the cells were put back on ice for another 5 min. Then 40 μl of 0.25 M EDTA (pH8.0) was added and the cells chilled another 5 min on ice. Cell lysis was completed with the addition of 1.6 ml Triton-X lytic solution (Section 3.5). The volume was brought up to 4 ml with TE and the solution centrifuged in a Beckman SW60 rotor at 15,000 RPM for 30 min to pellet the cell debris. The cleared lysate was transferred to a clean tube and stored at -20°C.

b) Isolation of the relaxation complex

200 μl of cleared lysate was loaded onto a 15-50% sucrose gradient.

Sucrose gradients were prepared by successively layering 0.5 ml of sucrose gradient buffer (50 mM Tris-HCl, 5 mM EDTA and 50 mM NaCl, pH 8.0) (Kline and Helinski, 1971) containing sucrose at concentrations decreasing at 5% increments into a centrifuge tube. The gradient was allowed to sit at room temperature for 2 hours prior to use. The samples were centrifuged in a Beckman SW60 rotor for 3 hr at 45,000 RPM. 300 μ l (15 drops) fractions were collected from the a needle inserted into the bottom of the tube. 30 μ l of each fraction was transferred to scintillation vials containing 10 ml of Scintiverse (Fisher Scientific) and counted on a scintillation counter. Complexed plasmid DNA was pooled from the fractions showing peak counts.

c) Relaxation assay

The complexed DNA sample was diluted with TE buffer a minimum of 5 fold to reduce the sucrose concentration to below 5% to allow the sample to be loaded onto the 5-20% sucrose gradient. 300 μ l aliquots of the test sample was transferred to Eppendorf tubes and 10 μ l of 30% SDS was added to one while 30 μ l TE buffer to the other and 65 μ l of Pronase (5 mg/ml) to a third. All tubes were incubated at 37°C for 30 min. Each sample was loaded onto a 5-20% sucrose gradient and centrifuged in a Beckman SW60 centrifuge at 45,000 RPM for 2 hr. 200 μ l fractions (10 drops) were collected from the bottom of the tube through a needle directly into 10 ml of scintillation fluid. The fractions were counted on a scintillation counter and the counts plotted against the fraction number.

4. RESULTS AND DISCUSSION

4.1. Localization of *oriT*

Prior to this study, pCU404 was the smallest pCU1 derivative which carried all of the *cis* acting functions necessary for conjugal transfer as demonstrated by its efficient mobilization by the parent plasmid (Thatte *et al.*, 1985). pCU404 contains the *Bgl*III fragment extending from 7 to 11.5 Kb on the pCU1 map (Figure 4.1). In addition to *oriT*, this 4.5 Kb *Bgl*III fragment also contains the right hand limit of the pCU1 *tra* region as defined by the Tn5 insertion in the self-transmissible, pCU1 derivative, pCU29 (Thatte *et al.*, 1983). The Tn5 insert in pCU29 has been mapped at 8.2 on the pCU1 map. Together, this information placed the *oriT* in the 1.2 Kb region extending from 7 to 8.2 Kb on the pCU1 map (Figure 4.1).

The proposed strategy to further localize the *oriT* of pCU1 was to subclone the smallest known fragment required in *cis* for transfer and then create deletions into the region with *Bal*31 nuclease.

4.1.1. Redefinition of the right hand limit of *tra*

Tn5 carries a *Bgl*III restriction site 1515 bps from each end, within the two IS50 sequences (Rothstein *et al.*, 1979) (Figure 4.1). These sites would allow the pCU1 *oriT* to be subcloned on a 2.7 Kb *Bgl*III fragment from pCU29. An attempt to shotgun clone this *Bgl*III fragment from pCU29 into the cloning vector pMK2004 indicated a mapping error in the reported location of the Tn5 insert in pCU29 and hence the right hand limit of *tra*.

Figure 4.1. Plasmid derivatives of pCU1 used in this study. Outer arcs represent regions cloned into vector DNA as follows: pCU404 (pMK2004), pCU56 (pACYC184), pCU101 (pACYC184), pCU109 (pACYC184) or in the case of pCU401 a deletion derivative of pCU1 carrying the pCU1 replicon (Thatte *et al.*, 1985₁, Thatte *et al.*, 1985₂). Ap^r, ampicillin resistance, Sp^r, spectinomycin resistance, Sm^r, streptomycin resistance, Rep, replication region, Tra, transfer region. Hatched (▨) portion of the *tra* region indicates the *oriT* region as determined in this study. Complementation regions of *tra*, designated by letters A-H, were defined by complementation tests between Tn5 insertion derivatives of pCU109 (Thatte *et al.*, 1985₁). Wavy borders (~~~~) indicate uncertainty in the extent of the complementation regions due to the lack of Tn5 insertions within the enclosed segment of DNA. Arrowheads (➤) identify the Tn5 insertions accompanied by the plasmid designation of the insertion derivative. In the case of pCU29 and pVT30, the placement of the Tn5 inserts represent corrected locations as determined in this study by *HpaI* digests. Tn5 has been expanded (heavy solid line) in the pVT30 derivative to identify relevant restriction sites within the IS50 elements at each terminus (inner thin line of Tn5). Restrictions sites are indicated: B, *Bam*HI, Bg, *Bgl*II, H, *Hind*III, Hp, *Hpa*I, K, *Kpn*I, P, *Pst*I.

pMK2004 (Kahn *et al.*, 1979) is a 5.2 Kb cloning vector which encodes a ColE1 type replicon similar to that found in the pBR322 cloning vector. Unlike pBR322, the ColE1 region containing *nic* has been deleted from pMK2004 making it more suitable for studies involving the *oriT* region of pCU1 (Covarrubias *et al.*, 1981). pMK2004 encodes resistance to three antibiotics: ampicillin, kanamycin and tetracycline. There are unique restriction sites within each of the antibiotic resistance genes which can be used for cloning and selection by insertional inactivation. In particular, insertions into the *Bam*HI site result in the inactivation of the tetracycline gene.

pCU29 DNA, digested with *Bgl*II, was ligated with *Bam*HI digested pMK2004 DNA and subsequently transformed into HB101. pCU1 was introduced into 200 Km^r Tc^s transformants by pellet mating. 40 Sp^r Km^r transconjugants were tested for the ability of the Km marker to be transferred during subsequent pellet matings with C600Nal. DNA was extracted from seven isolates demonstrating mobilization and analyzed by restriction digestion. Because the ligation of *Bgl*II and *Bam*HI sites results in the destruction of both sites, the *Bgl*II restriction pattern of the recombinant plasmid could not be compared with that of pCU29. Instead the clones were digested with *Hpa*I restriction endonuclease. The 2.7 Kb *Bgl*II fragment carrying the *oriT* region contains two *Hpa*I sites: one within the Tn5 derived DNA, 185 bp from its terminus and a second within the pCU1 derived DNA, at approximately 7.8 Kb on the pCU1 map (see Figure 4.1). A Tn5 insertion at 8.2 Kb should produce a 0.6 Kb *Hpa*I fragment within the *Bgl*II fragment carrying *oriT*.

The *Hpa*I restriction pattern of one of the clones, pBB91, seen in Figure 4.2 is representative of all seven isolates. These clones did not possess a 0.6 Kb *Hpa*I

Figure 4.2. Comparison of *Hpa*I restriction patterns of pCU29 (lane 2), pBB91 (lane 3), pVT30 (lane 4) and pSP1 (lane 5). Lane 1 contains 1 Kb DNA size standard (BRL). The 1.95 Kb fragment of pCU29 and pBB91 and the 1.55 Kb fragment of pVT30 and pSP1 carrying the pCU1 *oriT* are indicated on the right hand side of the photo.

restriction fragment predicted by a Tn5 insert at 8.2 on the pCU1 map. Instead, the *HpaI* digest produced a 1.95 Kb fragment, suggesting that the Tn5 insert of pCU29 should more accurately be placed at approximately 9.55 Kb on the pCU1 map (1.75 Kb from the *HpaI* site at 7.8). Accordingly, the region containing *oriT* was revised to lie between co-ordinates 7 and 9.55 Kb on the pCU1 map (Figure 4.1).

4.1.2. Subcloning the *oriT* region from pVT30: construction of pSP1

With the realization that the Tn5 insertion in pCU29 was 1.35 Kb from its reported position, a search was made for other Tra⁺ Tn5 insertion derivatives carrying the transposon closer to the *oriT* region of pCU1.

pCU109 is a self-transmissible, recombinant plasmid carrying the pCU1 *tra* region on a *HindIII* fragment cloned from pCU88 (Tn5 insert into pCU1 at approximately 11.2 Kb) in the single *HindIII* site on the cloning vector pACYC184 (see Figure 4.1) (Thatte *et al.*, 1985,). pVT30 is one of a series of Tra⁺ Tn5 derivatives of pCU109 with the Tn5 insert mapped at approximately 8.8 Kb on the pCU1 map (Thatte *et al.*, 1985,). pVT30 was used to construct an *oriT* containing recombinant plasmid pSP1, which could subsequently be used to construct deletions into the *oriT* region. Figure 4.3 outlines the construction of pSP1.

pVT30 plasmid DNA, digested with *BglII*, was ligated with pMK2004 plasmid DNA linearized at the unique *BamHI* site. The ligation mixture was transformed into HB101 and Km^r transformants were screened for inactivation of the tetracycline resistance gene. pCU1 was introduced into 12 of the Km^r Tc^s transformants (designated pBB11-22) by 'pellet matings' with C600/pCU1. Sp^r Km^r transconjugants were purified and mobilization experiments conducted by a second pellet mating with

Figure 4.3. Construction of pSP1 and pSP3. The 4.5 Kb *Bgl*II fragment containing the right hand end of pCU1 *tra* was subcloned from pVT30 into the unique *Bam*HI site of vector pMK2004. Tn5 derived *Pvu*II-*Hpa*I fragment was removed from pSP1 via partial digestion with both enzymes and recircularized to produce pSP3. In pSP3, the pCU1 derived *Pvu*II₁-*Hpa*I₂ fragment has been inverted with respect to pSP1. pMK2004 DNA is signified by a single line, pCU1 DNA from pVT30 by a double line, and Tn5 DNA from pVT30 by a solid heavy line. Plasmids have not been drawn to scale relative to each other, however regions within each plasmid are drawn to scale within the limitations of restriction digests. Regions conferring phenotypic characteristics are indicated by arcs within the circles Tra, transfer region, Ap^r, ampicillin resistance, Tc^r, tetracycline resistance, Km^r, kanamycin resistance, Rep, replicon. Restriction site designations B, *Bam*HI, Bg, *Bgl*II, E, *Eco*RI, H, *Hind*III, Hp, *Hpa*I, PvuI, *Pvu*II, P, *Pst*I, S, *Sal*I. *Pvu*II sites of pVT30 are numerous, therefore, they have only been identified in pSP1 and pSP3 derivatives.

C600NaI.

One of the four isolates demonstrating mobilization of the kanamycin marker at frequencies comparable to pCU404 was examined by restriction analysis. As seen in Figure 4.2, the *Bgl*II fragment sub-cloned from pVT30 contained a 1.55 Kb *Hpa*I fragment comparable to one of the bands in the pVT30 *Hpa*I digest. This places the Tn5 insert at 9.15 Kb on the pCU1 map, 400 bps closer to the right hand limit of *tra* than the Tn5 insert in pCU29. This derivative was named pSP1.

4.1.3. Construction of pSP3

pSP1 was constructed to obtain a derivative which could be subjected to *Bal*31 digestion from unique restriction sites (*Eco*RI or *Sal*I) on either side of the region containing *oriT* creating a series of nested deletions extending into the *oriT* region. *Bal*31 nuclease is a double stranded exodeoxyribonuclease which digests DNA from both ends of linear double stranded DNA. Because digestion occurs at a comparable rate from either end of linear DNA (Gray *et al.*, 1975) the distance between the unique *Sal*I site and the pCU1 derived DNA in pSP1 was prohibitive to obtaining deletions to the distal end of the pCU1 DNA without also deleting the plasmid replicon. As a result steps were taken to delete the intervening Tn5 derived *Pvu*II-*Hpa*I fragment from pSP1 (see Figure 4.3).

pSP1 DNA was digested with limiting concentrations of *Hpa*I and *Pvu*II restriction endonucleases for 30 min at 37°C in order to obtain partially digested fragments. A portion of this digest was then self-ligated and transformed into HB101. pCU1 was introduced into a total of 219 Km^r transformants by pellet matings and the resultant Sp^r Km^r transconjugants were tested for mobilization of the Kanamycin

Figure 4.4. Comparison of pSP1 and pSP3 restriction patterns. Comparisons of the *Pst*I (lanes 2 and 3) and the *Hind*III (lanes 4 and 5) digests of the two plasmids, demonstrate the loss of 1.2 Kb as well as one *Hind*III and one *Pst*I site in pSP3 with respect to pSP1. Comparison of the *Hind*III + *Hpa*I digests (lanes 6 and 7) demonstrates the inversion of the pCU1 derived *Pvu*II₁-*Hpa*I₂ fragment in pSP3 with respect to pSP1. The inversion results in a 2.27 Kb fragment rather than the 1.57 Kb fragment seen in pSP1. Lane 1 contains the 1 Kb DNA ladder (BRL).

lane 2	lane 3	lane 4	lane 5	lane 6	lane 7
pSP1 <i>Pst</i> I	pSP3 <i>Pst</i> I	pSP1 <i>Hind</i> III	pSP3 <i>Hind</i> III	pSP1 <i>Hind</i> III + <i>Hpa</i> I	pSP3 <i>Hind</i> III + <i>Hpa</i> I
5.13	8.21	4.10	5.63	2.76	3.36
<u>4.35</u>		2.76	<u>2.59</u>	2.59	2.59
9.48		<u>2.59</u>	8.22	1.57 ^a	<u>2.27</u>
		9.45		<u>1.00</u>	8.22
				9.49	

^a doublet.

marker by pellet matings with C600Nal. 50 transconjugants demonstrating mobilization frequencies comparable to pCU404 were screened by Eckhardt gel electrophoresis to group the clones on the basis of size.

Restriction analysis on representatives of each group identified one clone, designated pSP3, which carried the desired deletion. The restriction patterns shown in Figure 4.4 demonstrate a decrease in size of 1.2 Kb in pSP3 relative to pSP1 as well as a loss of one *HindIII* and one *PstI* site corresponding to the deletion of the Tn5 *HpaI*-*PvuII* fragment. Further restriction analysis using combined *HindIII*/*HpaI* (Figure 4.4) and *EcoRI*/*PvuII* digests (not shown), demonstrated that the pCU1 derived *HpaI*-*PvuII* fragment had inverted in pSP3 such that the pCU1 derived *HpaI* site was 2.3 Kb from the zero co-ordinate *HindIII* site rather than 1.5 Kb as seen in pSP1. A detailed restriction map of pSP3 is given in Figure 4.5.

4.1.4. Bal31 deletion mutagenesis of pSP3

pSP3 DNA was digested to completion by *EcoRI*, *Sall* or *PvuII* in separate reactions. Each sample of linear DNA was digested with Bal31 at 30°C over a range of incubation times of 2 to 10 minutes. Each set of timed reactions was pooled, the overhang ends were filled in with Klenow polymerase and *Bam*HI linkers were ligated to the ends. The DNA was recircularized and transformed into HB101. The resulting transformants were designated pBP, pBE and pBS depending on the restriction site at which pSP3 was linearized prior to Bal31 digestion. pBE transformants were selected on kanamycin plates while pBS and pBP transformants were selected on ampicillin plates.

Figure 4.5. Detailed restriction map of pSP3. See Figure 4.3 for symbols. D, *Dra*I, R, *Rsa*I, Ss, *Ssp*I, X, *Xmn*I.

Rapid plasmid DNA extractions from 75 pBP and 50 pBS isolates were digested in a double *EcoRI/BamHI* reaction to determine the extent of the *Bal31* digestion. Confirmation was obtained with *HindIII/BamHI* digests. 70 pBE transformants were screened by *PvuII/BamHI* digests and deletions were confirmed with *HindIII/BamHI* digests. A group of isolates representing a series of increasing deletions into either end of the pCU1 *oriT* region were kept for further study. A photograph of the electrophoresis gel of the *EcoRI/BamHI* or *PvuII/BamHI* digests can be seen in Figure 4.6. The extent of the deletions obtained are represented in Figure 4.7.

pCU101, a *Tra*⁺ derivative of pCU1 (*HindIII* fragment from pCU29 cloned into the *HindIII* site of vector pACYC184, Thatte *et al.*, 1985₁), was transferred into each of the chosen *Bal31* deletion derivatives via pellet matings. Mobilization of each deletion derivative was determined by large filter matings with recipient C600Nal. The transfer frequency of the *Bal31* derivative with respect to the transfer frequency of the self-transmissible plasmid pCU101 is also indicated in Figure 4.7. In the case of deletions extending from the *PvuII* or *SalI* site, all isolates with *EcoRI/BamHI* fragments 1.4 Kb or larger were *Mob*⁺ while those with a corresponding fragment of less than 1.4 Kb are *Mob*⁻. In the case of deletions extending from the *EcoRI* site of pSP3, derivatives carrying a *PvuII/BamHI* fragment of 1.7 Kb or less were *Mob*⁻ while those with corresponding fragments greater than 2.2 Kb were *Mob*⁺.

Plasmids pBP1 and pBE60 represent the largest deletion extending into either end of the *oriT* region which are still mobilized by pCU101 at frequencies comparable to pCU404. By aligning the pBP1 and pBE60 deletion derivatives it can be concluded that the region required in *cis* for transfer is encoded within the overlap of

Figure 4.6. Gel electrophoresis of pSP3 Bal31 deletion derivatives. Plasmid DNA was digested in a double digest to determine the size of the deletion into the *oriT* region. Lanes 2-12, *EcoRI/BamHI* digests of pBP and pBS derivatives. Lanes 14-18, *PvuII/BamHI* digests of pBE derivatives. In each instance the lower band represents the fragment containing *oriT* DNA while upper band represents vector DNA. Lane 1 and 13, 1 Kb DNA size standard.

Lane	plasmid	size of <i>oriT</i> fragment (Kb)
2	pSB47	1.13
3	pBP11	1.28
4	pBP1	1.41
5	pBP50	1.41
6	pBS8	1.55
7	pBS17	1.71
8	pBS18	1.82
9	pBS32	1.95
10	pBS6	2.08
11	pBS22	2.08
12	pBS38	2.29
14	pBE48	1.58
15	pBE26	1.61
16	pBE42	1.71
17	pBE60	2.18
18	pBE44	2.53

Figure 4.7. Schematic representation of pSP3 Bal31 deletion derivatives and their corresponding mobilization frequencies by pCU101. Upper line represents pSP3 where open double line represents pCU1 DNA, solid heavy line represents Tn5 DNA and single line represents pMK2004 vector DNA. Lower lines represent Bal31 deletion derivatives obtained by Bal31 digestion of pSP3 linearized at either the *Sa*I (pBS derivatives), *Pvu*II (pBP derivatives) or *Eco*RI (pBE derivatives) sites followed by the addition of *Bam*HI linkers (B) to filled in termini and recircularization. Gaps indicate regions deleted from pSP3 in each derivative. Mobilization frequencies were determined by large filter matings and represent the number of transconjugants receiving the *oriT* derivative per transconjugant receiving the helper plasmid pCU101. The transfer frequency of pCU101 ranged from 1.3×10^{-2} to 7.3×10^0 . See figures 4.3 and 4.4 for symbols.

Derivative	Mobilization frequency by pCU101
pSP3	7.2×10^{-1}
pBS47	5.2×10^{-6}
pBP11	4.1×10^{-6}
pBP1	2.6×10^{-1}
pBP50	1.5×10^{-1}
pBS8	2.0×10^{-1}
pBS17	1.3×10^{-0}
pBS18	9.7×10^{-1}
pBS32	1.2×10^0
pRS6	2.7×10^0
pBS22	2.9×10^0
pBS38	3.0×10^0
pBE48	9.3×10^{-4}
pBE26	1.9×10^{-4}
pBE42	5.9×10^{-4}
pBE60	1.0×10^0
pBE44	1.0×10^0

the two derivatives. The deletions in three other constructs pBP11, pBS47 and pBE42, can be seen to extend into but not beyond this overlap region. The frequency of mobilization for each of these three constructs is significantly reduced with respect to pBP1 and pBE60. These five plasmids were subsequently used to sequence the overlap in pBE60 and pBP1 containing the *oriT* region.

DNA sequence analysis, which will be described in Section 4.2, identified several useful restriction sites within the *oriT* region. In particular two *SspI* sites and single *RsaI*, *DraI*, *SnaBI* and *BspMI* sites were used to construct additional deletions within the *oriT* region carried by pBP1 and pBE60.

4.1.5. Construction of deletions within pBP1 and pBE60

Eleven derivatives constructed from pBP1 are illustrated in Figure 4.8, while the two constructed from pBE60 are illustrated in Figure 4.9. An expanded view of the *oriT* region carried by each derivative is presented in Figure 4.10. The two *SspI* restriction sites within the 692 bp overlap of pBP1 and pBE60 divide it into three regions of comparable size (Figure 4.10). Derivatives pSP11-16 have had one or more of these *SspI* regions deleted. pSP15 which has lost the internal *oriT* *SspI* fragment is comparable to a derivative, pSP17 which was constructed by ligating the *BamHI-HindIII* fragments from pBE42 and pBS47 (see Figure 4.7 and 4.10). Within the internal *SspI* region, are three other restriction sites, *SnaBI*, *BspMI* and *DraI*. Derivatives pSP18-23 have had the region to one side of these sites deleted. In derivative pSP25 the sequence to one side of the *RsaI* site has been deleted.

Figure 4.8. Construction of deletions within pBP1. Inner circle is pBP1. Outer arcs represent the deletion derivatives. Gaps indicate the region of pBP1 deleted from each derivative. See Figure 4.5. for symbols. The *oriT* region (double line) contains two *SspI* sites. Partial digestion of pBP1 with *SspI*, followed by religation and transformation of HB101 produced five derivatives designated pSP11-15 containing different *oriT SspI* fragments. Derivative pSP16, which contains only the internal *oriT SspI* fragment, was constructed by an *SspI* deletion of derivative pSP12.

Within the internal *SspI* fragment, there are three restriction sites, *DraI*, *SnaBI* and *BspMI*. Three derivatives, pSP18, pSP19 and pSP20 were constructed by deleting the region counterclockwise of the *oriT DraI*, *SnaBI* or *BspMI* sites respectively. pBP1 DNA was digested with either *DraI*, *SnaBI/XmnI* or *BspMI/XmnI* and recircularized. pSP21 was created by ligating the *SnaBI* and *BspMI* site of pSP12 together resulting in a deletion of the intervening 65 bp. In all ligations involving the *BspMI* site it was necessary to fill in the overhang ends created by the *BspMI* digestion to produce blunt ends prior to ligation.

pSP25 was constructed by partial *RsaI* digestion of pBP1 DNA followed by recircularization. The pCU1 *oriT* region (double line) has been expanded in Figure 4.10.

Figure 4.9. Construction of deletions in pBE60. Inner circle is pBE60. Outer arcs represent the deletion derivatives. Gaps indicate the region of pBE60 deleted from each derivative. See Figure 4.5 for symbols. pSP22 and pSP23 were created by deleting the *oriT* region clockwise from the *Sna*BI and *Bsp*MI sites respectively. Both plasmids were constructed from pBE60 by ligating the *Sna*BI or *Bsp*MI site to the *Hpa*I site. The pCU1 *oriT* region (double line) has been expanded in Figure 4.10.

Figure 4.10. Expanded pCU1 *oriT* region showing the extent of deletions into each *oriT* derivative. The 830 bp sequenced region (co-ordinates 8.5-9.3 Kb on the pCU1 map, Section 4.2) is indicated by the heavy line. The region above the line identifies the direct repeats, $\longleftrightarrow$, inverted repeat, $\rightarrow\leftarrow$, and the Integrative Host Factor (IHF) recognition sequences $-$. Below the line, the extent of deletion of each *oriT* derivative is indicated. The number at the end of each line indicates the terminal nucleotide number determined from the sequence in Figure 4.13. See Figure 4.8 for symbols.

4.1.6. Mobilization of *oriT* derivatives

Initially, pCU101 was transferred into each of the *oriT* deletion derivatives by pellet matings and the mobilization frequencies determined by large filter matings with C600NaI. Due to apparent instability of the pCU101 derivative, these mobilization experiments were repeated with pCU1 or pCU401 as the conjugative helper instead of pCU101. pCU401 (Figure 4.1) is a pCU1::Tn5 derivative from which the Ap, Sm and Sp resistance genes have been deleted leaving only the Km resistance gene for selection (Thatte *et al.*, 1985₂). pCU401 was used in place of pCU1 when the *oriT* derivative carried only the Ap resistance gene of the pMK2004 vector to allow for antibiotic selection of each plasmid marker. The results of these mobilization experiments are presented in Tables 4.1 and 4.2 respectively.

On comparison of the results obtained with the different Tra⁺ helper plasmids, (second column, Table 4.1 and 4.2) it is apparent that the transfer ability of the pCU101 derivative is reduced with respect to pCU1 or pCU401 (an average frequency of 0.4 transconjugants per donor compared with 7.2 or 1.4 transconjugants per donor). The cause of the reduced mobility with pCU101 may be related to the segregational instability seen with this plasmid. This will be discussed in further detail below. Reduced mobility is seen to a lesser extent with the pCU401 derivative which transferred at a frequency of 1.4 transconjugants per donor. Although pCU1 is able to transfer itself at a higher frequency than pCU101, the relative mobilization frequency of the non-conjugative plasmids with respect to the transfer frequency of the conjugative plasmid remains the same in most cases. However, there is disagreement between the two sets of mobilization experiments

Table 4.1. Mobilization frequency of pCU1 *oriT* derivatives by conjugative plasmid pCU101.

<i>oriT</i> plasmid ^a	pCU101 (Cm ^r) transfer frequency ^b	<i>oriT</i> plasmid (Km ^r) transfer frequency ^b	mobilization frequency ^c	Cm ^r /Km ^r ^d
pCU404	2.1x10 ⁻¹	1.8x10 ⁻²	8.6x10 ⁻²	27/200
pBP1	2.7x10 ⁻¹	1.3x10 ⁻²	4.8x10 ⁻²	165/309
pBE60	9.0x10 ⁻¹	9.4x10 ⁻¹	1.0x10 ⁰	59/110
pSP25	2.5x10 ⁻¹	1.8x10 ⁻¹	7.2x10 ⁻¹	ND
pSP12	7.3x10 ⁻¹	1.6x10 ⁻²	2.2x10 ⁻²	84/321
pSP19	2.7x10 ⁻¹	6.3x10 ⁻⁴	2.3x10 ⁻³	149/200
pSP20	2.6x10 ⁻¹	8.5x10 ⁻⁵	3.3x10 ⁻⁴	5/109
pSP21	1.7x10 ⁻¹	4.1x10 ⁻⁵	2.4x10 ⁻⁴	0/196
pBP11	1.8x10 ⁻¹	7.5x10 ⁻⁷	4.2x10 ⁻⁶	11/106
pSP14	3.2x10 ⁻¹	<2.0x10 ⁻⁶	<6.3x10 ⁻⁶	5/13
pBE42	2.9x10 ⁻¹	1.7x10 ⁻⁶	5.9x10 ⁻⁶	0/156
pBS47	5.4x10 ⁻¹	2.8x10 ⁻⁶	5.2x10 ⁻⁶	ND
pSP11	5.0x10 ⁻¹	<2.2x10 ⁻⁷	<4.4x10 ⁻⁷	0/8
pSP13	6.1x10 ⁻¹	<4.0x10 ⁻⁶	<6.6x10 ⁻⁶	0/22
pSP15	4.0x10 ⁻¹	4.7x10 ⁻⁷	1.2x10 ⁻⁶	0/31
pSP16	2.2x10 ⁻¹	<1.2x10 ⁻⁷	<5.5x10 ⁻⁷	0/1
pSP17	2.4x10 ⁻¹	4.9x10 ⁻⁷	2.0x10 ⁻⁶	0/18
pSP18	8.5x10 ⁻¹	<5.4x10 ⁻⁸	<6.4x10 ⁻⁸	0/5
pSP22	2.8x10 ⁻¹	4.3x10 ⁻⁶	1.5x10 ⁻⁵	0/200
pSP23	4.0x10 ⁻¹	3.8x10 ⁻⁵	9.5x10 ⁻⁵	0/200
pMK2004	3.3x10 ⁻¹	2.6x10 ⁻⁶	7.9x10 ⁻⁶	0/126

^a *oriT* derivatives were maintained in HB101 with pCU101

^b number of recipients receiving the plasmid's antibiotic marker per donor.

^c ratio of recipients receiving the *oriT* derivative per recipient receiving pCU101

^d number of transconjugants receiving the *oriT* derivative (Km^r) which did not co-inherited pCU101 (Cm^r).

ND not done

Table 4.2. Mobilization frequency of pCU1 *oriT* derivatives by conjugative plasmids pCU1 or pCU401.

<i>oriT</i> plasmid ^a	pCU1 (Sp ^r) or pCU401 (Km ^r) transfer frequency ^b	<i>oriT</i> plasmid (Km ^r or Ap ^r) transfer frequency ^b	mobilization frequency ^c	Sp ^r /Km ^r or Km ^r /Ap ^r ^d
pCU404	1.8x10 ¹	5.1x10 ⁰	2.8x10 ⁻¹	2/200
pBP1	6.2x10 ⁰	3.7x10 ⁰	6.0x10 ⁻¹	2/300
pBE60	1.5x10 ¹	4.9x10 ⁰	3.3x10 ⁻¹	1/200
pSP25	2.2x10 ⁰	3.8x10 ⁻¹	1.7x10 ⁻¹	ND
pSP12	6.8x10 ⁰	3.5x10 ⁻¹	5.1x10 ⁻²	0/257
pSP19	2.9x10 ⁰	4.5x10 ⁻³	1.6x10 ⁻³	0/285
pSP20	2.4x10 ⁰	1.7x10 ⁻⁵	7.1x10 ⁻⁶	0/160
pSP21	6.6x10 ⁻¹	1.1x10 ⁻⁵	1.7x10 ⁻⁵	ND
pBP11	1.1x10 ¹	3.8x10 ⁻⁵	3.5x10 ⁻⁶	0/200
pSP14*	3.8x10 ⁰	3.4x10 ⁻⁵	8.9x10 ⁻⁶	7/30
pBE42	6.7x10 ⁰	1.8x10 ⁻³	2.7x10 ⁻⁴	0/100
pBS47*	2.0x10 ⁻¹	2.5x10 ⁻⁶	1.3x10 ⁻⁵	0/17
pSP11	3.0x10 ¹	8.9x10 ⁻⁶	3.0x10 ⁻⁷	0/185
pSP13*	1.1x10 ⁰	1.9x10 ⁻⁵	1.7x10 ⁻⁵	0/32
pSP15	1.2x10 ⁰	4.8x10 ⁻⁵	4.0x10 ⁻⁵	0/150
pSP16*	4.5x10 ⁻¹	<5.5x10 ⁻⁷	<1.2x10 ⁻⁷	0/1
pSP17	1.6x10 ⁰	2.9x10 ⁻⁵	1.8x10 ⁻⁵	1/150
pSP18	6.5x10 ⁰	7.6x10 ⁻⁶	1.2x10 ⁻⁶	0/200
pSP22	3.0x10 ⁰	9.9x10 ⁻⁶	3.3x10 ⁻⁶	ND
pSP23	2.6x10 ⁰	8.9x10 ⁻⁷	3.4x10 ⁻⁷	ND
pMK2004	1.5x10 ⁰	3.2x10 ⁻⁵	2.1x10 ⁻⁵	0/200

^a *oriT* derivatives were maintained in HB101 with either pCU1 or pCU401. * indicates those strains carrying the conjugative plasmid pCU401, all others carried pCU1.

^b number of recipients receiving the plasmid's antibiotic marker per donor.

^c ratio of recipients receiving the *oriT* derivative per recipient receiving pCU1 or pCU401.

^d number of transconjugants receiving the *oriT* derivative (Km^r or Ap^r) which did not co-inherited pCU1 (Sp^r) or pCU401 (Km^r)

ND not done

with *oriT* derivatives pBE42, pSP20, pSP21, pSP22 and pSP23. In the first instance mobilization of the *oriT* derivative is 100 fold more efficient by pCU1 whereas the other derivatives are mobilized 100 fold more efficiently by pCU101.

Based on the mobilization frequencies by the parent plasmid pCU1, the 19 *oriT* derivatives tested can be divided into two groups. The first group consists of those derivatives which are mobilized at frequencies greater than the frequency observed with the vector pMK2004 (10^{-5}) and includes pBP1, pBE60, pSP25, pSP12 and pSP19. The elevated mobilization frequency is an indication that these derivatives are donated by pCU1 and contain at least some of the *cis* acting *tra* functions. Other experiments (section 4.17) have confirmed this. Within this group, pBP1 and pBE60 are mobilized at frequencies comparable to pSP3 or pCU404 indicating that they contain all of the *cis* acting sequences necessary for conjugal transfer. As DNA is deleted from the left hand end of the pBP1-pBE60 overlap region, the mobilization frequency decreases. The reduced mobilization frequencies of pSP25, pSP12 and pSP19 suggests that some of the region required in *cis* for transfer has been deleted from these derivatives.

Due to the relatively small drop in mobilization frequency observed with pSP25 as compared to pBP1, pBE60, pSP3 or pCU404, statistical analysis of variance was conducted to determine if the variation observed between the mean mobilization frequencies was significant. The results (see Figure 4.11) indicated that while the average mobilization frequency of pBE60 did not differ significantly at the 95% confidence level, from the parental plasmids pSP3 or pCU404, the average frequencies for both pBP1 and pSP25 did differ, pBP1 being significantly higher than pSP3, pCU404 and pBE60 while pSP25 was significantly lower. Since pBP1 is a

Figure 4.11. Analysis of variance of mobilization means.

Formula:

$$F = \frac{\left(\sum \frac{T_i^2}{n_i} - \frac{T^2}{n} \right) / (k-1)}{\left(\sum T_i^2 - \frac{T^2}{n} \right) / (n-k)}$$

where T_i is the sum of all observations in sample i .

T is the sum of all observations.

n_i is the number of observations in sample i .

n is the total number of observations

k is the number of samples.

Mobilization frequency^a of pCU1 *oriT* derivatives by pCU1

pBP1	pCU404	pSP3	pBE60	pSP25 ^b		
5.14	2.35	3.78	2.62	1.08		
5.24	4.64	3.82	2.81	1.15		
5.30	4.75	3.83	3.20	1.36		
5.54	5.62	4.13	3.82	1.41		
5.55	6.23	5.13	4.13	1.49		
6.14			4.46	1.55		
8.37			5.08	1.67		
8.91			5.11	1.78		
10.9				1.84		
				2.42		
				2.45		
Comparison				Calculated F value	95% F value ^c	
pBP1=pCU404=pSP3=pBE60=pSP25				20.56	>2.69	reject
pBP1=pCU404=pSP3=pBE60				6.61	>3.03	reject
pCU404=pSP3=pBE60=pSP25				20.67	>2.99	reject
pCU404=pSP3=pBE60				0.94	<3.68	accept

^a includes values which are not included in Table 4.2

^b includes values from mating with comparable construct pSP26 in which the *RsaI*-*Bam*HI fragment from pSP12 was subcloned into the *Bam*HI site of pMK2004.

^c taken from Remington and Schork (1970).

deletion derivative of pSP3 yet has a higher mobilization frequency, these findings suggest that factors other than the amount of DNA carried by a plasmid can have significant effects on the mobilization efficiency. These findings make it difficult to clearly state whether the slight reduction in pSP25 mobilization frequency is indeed due to loss of necessary functions or to factor(s) which cause pSP3 and pCU404 (both of which carry more pCU1 DNA than pBP1) to have statistically lower transfer frequencies than pBP1.

The second group of *oriT* derivatives are characterized by mobilization frequencies comparable to the negative control, pMK2004 ($<10^{-5}$). The low mobilization frequencies of these derivatives are consistent with a belief that they are mobilized by conduction rather than by donation. However, there is the possibility that some of these low mobility derivatives are being donated at very low frequencies, rather than being conducted. This possibility will be considered in Section 4.4.

4.2. Sequence analysis of the *oriT* region

As indicated in the Introduction, the intent of this study was to begin an analysis of the *oriT* region of pCU1 and in the previous section, it was shown that this region could be isolated. For any further study of this region, knowledge of its nucleotide sequence would be useful. Experiments that established this sequence are described below.

2 of/de 2

MICROCOPY RESOLUTION TEST CHART
NATIONAL BUREAU OF STANDARDS
STANDARD REFERENCE MATERIAL 1010a
(ANSI and ISO TEST CHART No. 2)

4.2.1. Cloning into M13

pCU1 DNA from the *Bal31* deletion derivatives p9P1, pBP11, pBS47, pBE42 and pBE60 was first subcloned into the polylinker of the M13 derivative mp18 or mp19 such that the *Bam*HI site marking the end of each deletion lay next to the sequencing primer.

pBE42 and pBE60 were each digested in a double *Bam*HI/*Sal*I digest and ligated into M13mp19 DNA digested with the same enzymes. The resultant white plaques (4 of each) were first examined for inserts by the rapid ss phage DNA gel electrophoresis procedure. All of the isolates tested showed reduced mobility. DNA was isolated from each of the isolates by the boiling miniprep method. Presence of the proper insert was confirmed by a double *Bam*HI/*Sal*I digest.

Repeated attempts to subclone the *Bam*HI/*Eco*RI fragment from pBP1 and pBP11 into M13mp18 proved unsuccessful as did attempts to subclone the *Bam*HI/*Pst*I fragments into M13mp19. The difficulty encountered in trying to subclone the *oriT* region from these two derivatives suggests that the sequence of the DNA itself (in particular the inverted repeats common to *oriT* regions) may have interfered with the ligation step.

4.2.2. Cloning into pIBI76

pIBI76 was derived from the pEMBL cloning vectors (Dente *et al.*, 1983, IBI, 1987). The polylinker sequence contains the same order of restriction sites as M13mp18; however, the primer region is located on the side opposite to that of the M13mp18 vector. The pEMBL cloning vectors are noted for the increased stability of

cloned fragments.

Each of pBP1, pBP11 and pBS47 DNA was digested in a double digest with *Bam*HI and *Eco*RI. In order to enhance the chances of subcloning the desired DNA fragment, the fragment was removed from the gel using the Gene Clean™ gel extraction technique. The digested DNA was run on a 0.7% TAE electrophoresis gel and the appropriate band removed from the gel using the Gene Clean procedure. This DNA was ligated to pBI76 DNA, cut with *Eco*RI and *Bam*HI and transformed into DH5αF'. Single stranded DNA from 24 white plaques from each ligation were screened for inserts by gel electrophoresis. In each set of 24, two isolates demonstrated reduce mobility and upon digestion of miniprep DNA, one isolate containing the pCU1 derived DNA was observed. The low fraction of plaques which carried insertions is consistent with the difficulties encountered in attempts to clone these fragments into M13 derivatives.

4.2.3. Single stranded DNA sequencing

Single stranded recombinant DNA isolated from each of M13mp19-E60, M13mp19-E42, pBI76-P1, pBI76-P11 and pBI76-S47 was sequenced as outlined in the Methods and Materials (Section 3.14). Two successful trials with each derivative were used to determine the nucleotide sequence of the region extending from the *Bam*HI site at the end of the pBE60 Bal31 derivative to the limit of sequence clarity of the pBE42 derivative. The sequences derived from the other three clones lay within this 830 bp region. Figure 4.12 outlines the relationship between the regions and the strand sequenced in each derivative. Due to the nature of the insertions in the sequencing vector, the strand sequenced in the pBP1, pBP11 and pBS47

Figure 4.12. Strategy for sequencing the pCU1 *oriT* region. The 830 bps sequenced is indicated by the heavy line. Each of the five *oriT* derivatives used for sequencing are indicated. Arrow indicates the direction of sequence. Opposite strands are denoted by the straight and wavy lines. See Figure 4.8 for restriction site symbols.

derivatives was different from that sequenced in the pBE42 and pBE60 derivatives. With this in mind nucleotides 1-358 and 695-830 were derived from one strand of a single clone, nucleotides 358-380 and 562-694 were derived from different strands of two different clones, and nucleotides 380-562 from the same strand of two different clones.

Figure 4.13 represents the sequence determined for the *oriT* region of pCU1. The first 23 bp of the region sequenced from pBE60 correspond to the first 23 bp of Tn5 (Auerswald *et al.*, 1979) indicating that the *Bal*31 deletion in this derivative did not reach the pCU1 DNA. Sequencing also indicated that the DNA subcloned from pBE60 into M13mp19 contained a 97 bp spontaneous deletion spanning nucleotides 263-359. The deletion appears to have occurred by homologous recombination between direct repeats 6 and 13. Whether the deletion occurred in pBE60 or in the M13mp19 subclone is unknown, however considering that pBE60 was maintained in the RecA⁻ derivative HB101, it is more likely to have occurred in the M13mp19 subclone while in RecA⁺ strain JM101. This derivative was not used in any further experiments.

The pCU1 *oriT* sequence presented in Figure 4.13 is identical to the sequence published by Coupland *et al.* (1987) for the plasmid pKM101 with two exceptions: nucleotides 276 and 277 are reversed and the CGCG (bps 498-502) are GCGGC in the pKM101 sequence (nucleotides 421-425). These differences were confirmed by sequences obtained by V. Thatte from independently prepared clones in this lab.

Within the 830 bp sequenced, several features can be identified. Upon first glance at the sequence it is apparent that like the *oriT* regions of the IncF plasmids

Figure 4.13. pCU1 *oriT* region sequence. The 930 bp sequence was determined from the five *oriT* derivatives as outlined in Figure 4.12. The first 23 nucleotides correspond to Tn5 sequences (Auserwald, 1979). Nucleotides varying from the sequence of R46 *oriT* region reported by Coupland *et al.* (1987) are in brackets (). The end of the deletion in each of the five *oriT* derivatives used in the sequencing is indicated by ↵ or ↴ with the plasmid designation above. The region between the two slashes // (nucleotides 263/264 and 368/369 indicates the region deleted from the pBE60 derivative used for sequencing. The two *SspI*, and single *DraI*, *BspMI*, *RsaI* and *SnaBI* restriction sites are indicated in bold lettering. Directly repeated sequences are indicated by the heavy arrows above the sequence. The first set of thirteen, 11 bp direct repeats extends from nucleotide 201 to 324. The second set of two, 14 bp repeats are located at nucleotide 421-434 and 468-481, bases showing lack of consensus are indicated by a dot. Inverted repeats and regions of two fold rotational symmetry are indicated by light arrows above the sequence and are located at nucleotides 375-394, 431-442, 453-469, 504-527, 563-587 and 588-612, mismatched nucleotides are indicated by a dot. The first nucleotide of putative n' recognition sequences are indicated. Sequences showing consensus with the IHF sequence of lambda are indicated by lines below the sequence, bases lacking consensus are indicated by a dot. Boxed sequences represent regions showing homology to the proposed *nic* regions of (a) ColE1 and CloDF13 or (b) pSC101. These regions of homology are expanded in Figure 4.14.

F, R1, R100 and ColB4, the region is AT-rich. The AT content of the 830 bp sequence approaches 63%. This percentage rises to 67% for the region carried by pSP25 where there is one string of 19 bp (453-472) containing only two GC residues and a second 24 bp string (524-596) completely composed of A and T residues. This latter AT string is within a 81 bp sequence (524-606) which is 79% AT's.

The most striking feature of the 830 bp sequenced in pCU1, are the thirteen, 11 bp direct repeats starting at nucleotide 202 and running to nucleotide 374. These direct repeats have a consensus sequence of TG(A/T)TGTA(A/C)TTT, the underlined nucleotides each varying in four of the repeats.

In addition to the set of 11 bp direct repeats, a second 14 bp repeat TTANNCCTTAACCT is located at nucleotides 421-434 and 467-481. This repeated sequence is within the 90 bp region between the first *SspI* site and the *BspMI* site.

Within the sequenced region there are five inverted repeat sequences, three large (10 bps) and 2 small (6 and 7 bps) and one 12 bp region of imperfect two fold rotational symmetry. All of these structures lie within the region carried by pSP25. One 10 bp perfect inverted repeat lies just to the left of the first *SspI* site, outside the region carried by pSP12 (nucleotides 374-395). The remaining two large imperfect inverted repeats lie in the region between the second *SspI* site and the end of the *Bal31* deletion found in pBP1, outside the region carried by pBP11 (nucleotides 566-587 and 588-611) while the smaller two inverted repeats lie within the internal *SspI* fragment (nucleotides 453-469 and 504-527). The 6 bp inverted repeat at 453-469 overlaps the second 14 bp direct repeat. The region of imperfect two fold rotational symmetry spans nucleotides 431-442 and overlaps the first 14 bp direct repeat.

Within the 830 bp region sequenced there are two sequences showing 11 of

12 nucleotide consensus to the IHF binding site of lambda phage (PyAANNNTTGAT(A/T)). Both putative IHF binding sites are located within the region carried by pSP25, right of the second *SspI* site at nucleotides 567-600 and 657-669. Both sites lie outside the region carried by pBP11 and the first site overlaps one of the two inverted repeat sequences in this region.

Within the 870 bps sequenced there are only two sequences showing consensus to the λ protein binding site sequence, one at either end at nucleotide 72 and nucleotide 761. Both have one mismatch to the consensus sequence. Neither are associated with an inverted repeat sequence and both lie outside the region carried by pSP25.

DNA analysis computer software Caltech TM was used to compare the pCU1 *oriT* sequence with the published *oriT* sequences of F (Thompson *et al.*, 1984), R100 (McIntire and Dempsey, 1987), R1-19 (Finlay *et al.*, 1986), ColB4-K98 (Finlay *et al.*, 1986) ColE1 (Bastida, 1978), CloDF13 (Snijders *et al.*, 1983), RSF1010 (Derbyshire and Willetts, 1987), R1162 (Brasch and Mayer, 1987), pSC101 (Willetts and Wilkins, 1984), R64 (Komano *et al.*, 1988) and RK2 (Guiney and Yakobson, 1983).

Coupland *et al.* (1985) identified a 31 bp sequence of pKM101 *oriT* which showed 22 bp identity (70% homology) with the *oriT* sequence of pSC101. This sequence spans nucleotides 576-607 of the pCU1 *oriT* region and is identified in Figure 4.13 and expanded in Figure 4.14. The sequence overlaps one half of one 10 bp inverted repeat sequence and 75% of a second. The site similar to the one proposed by Nordheim *et al.* (1980) as the location of the pSC101 *nic* site falls within the base of the second inverted repeat. This region also spans the first putative IHF recognition sequence.

Figure 4.14. Homology between sequences of the pCU1 *oriT* region and the *oriT* regions of ColE1, CloDF13 and pSC101. The first nucleotide of the sequence is indicated in the second column and is as reported by Willetts and Wilkins, 1983. The sequences are presented in column three, nucleotides not showing identity to pCU1 are indicated by asterisks below the sequence. The two nucleotides inserted into ColE1 sequence to extend the homology are indicated by N. Inverted repeat sequences and regions of dyad symmetry are indicated by light arrows above the sequences, direct repeat sequences by heavy arrows above the sequence and putative IHF recognition sequences are indicated by lines below the sequences. Dots in these features indicate a lack of identity. The *nic* site proposed for ColE1 (Lovett *et al.*, 1974) and pSC101 (Nordheim, 1980) are indicated by arrows ▲ . The fraction of nucleotides showing consensus to pCU1 sequence is indicated in column four and the percent homology is indicated in column five.

A second case of sequence homology can be observed between the *oriT* regions of pCU1, ColE1 and CloDF13. Although the length of sequences showing homology are shorter than that observed with pSC101, the degree of homology is as great or greater. In both ColE1 and CloDF13, *nic* is believed to lie within a 11 or 12 bp region of imperfect two fold rotational symmetry which bear 7 bp identity to each other. These two sequences also bear striking resemblance to the sequences surrounding the two 14 bp direct repeats in the pCU1 *oriT*. In Figure 4.14 the two directly repeated sequences of pCU1 are aligned with the regions of two fold rotational symmetry of ColE1 and CloDF13.

Within the region surrounding the first direct repeat of pCU1 beginning at nucleotide 421, pCU1 and CloDF13 show 14/21 (66%) nucleotides consensus. The homology between pCU1 and ColE1 is not immediately obvious; however, the insertion of two nucleotides in ColE1 results in 15/21 (71%) nucleotides aligning. It should be noted that in both cases the 21 bps showing homology include the entire two fold rotational symmetry sequence of pCU1 allowing one to propose this region as a possible location of *nic*.

The region surrounding the second 14 bp direct repeat sequence of pCU1 beginning at nucleotide 467 also shows homology with the regions of dyad symmetry of ColE1 and CloDF13. In the case of ColE1, 12/16 (75%) nucleotides show identity with part of the ColE1 symmetry repeat and the region directly to the right while 11/14 (79%) nucleotides match the dyad sequence of CloDF13 and the nucleotides immediately to the left. Although this region of pCU1 does not encode a region of two fold rotational symmetry as found with the first direct repeat, the first 3 nucleotides of the direct repeat overlaps a 6 bp inverted repeat sequence. This

overlap is included in the region showing homology to CloDF13 but not ColE1. Also the overlap in CloDF13 does not include any part of the region of rotational symmetry of CloDF13.

4.3. Attempts to identify a pCU1 relaxation complex

The assumption that conjugal transfer of plasmids encoding an N *tra* system proceeds in the same general fashion as for those encoding an F *tra* system prompted the search for a SDS induced *in vitro* nicking event in pCU1 comparable to that found in F, ColE1, R100, RK2, R6K and R64. If such an event were detectable, relaxation tests on the various *oriT* derivatives constructed in section 4.2 combined with the sequence determined in the previous section (section 4.3), would enable placement of the *nic* site.

The procedure outlined in section 3.15 of the Methods and Materials section was tested with the plasmid pBRK4013 a high copy number derivative carrying the *tra* system of the IncP plasmid RK2. The results (Figure 4.15a) demonstrate that cleared lysate DNA from the peak of the 15-50% sucrose gradient (complexed DNA) run on a 5-20% sucrose gradient produced a single DNA peak while the same lysate treated with 0.25% SDS for 30 min at 37°C produced two DNA peaks, one similar to that found in the untreated sample and a slightly slower sedimenting band representative of relaxed DNA.

The same procedure was carried out on a strain carrying either pCU101 or pCU109. The results presented in Figure 4.15b and 4.14c demonstrated no significant difference between the untreated cleared lysate or the lysate treated with

Figure 4.15a-c. Relaxation of pBRK4013 and pCU1 derivatives. 5-20% sucrose gradient of relaxation complex preparations. Fractions from the bottom to the top of the sucrose gradient are plotted from left to right. 300 μ l of cleared lysate prepared as outline in the Methods and Materials, untreated, treated with 1% SDS at 37°C for 30 min. Gradients were centrifuged in a Beckman SW60 rotor for 2 hr.

0.25% SDS for 30 min at 37°C. The cleared lysate of pCU101 was also exposed to 1.25 mg/ml pronase at 37°C for 30 min. but no relaxation was observed (Figure 4.15d).

The gel electrophoresis method described by Thompson *et al.* (1989) also failed to demonstrate relaxation of pBP1 in the presence of pCU101. Additional confirmation is provided by the fact that strains carrying pBP1 alone and those carry pBP1 and pCU101 produce similar pBP1 bands on Eckhardt gels despite lyses by SDS within the gel wells (data not shown). Thus, *oriT* plasmids derived from pCU1 do not appear to form relaxation complexes under conditions shown to relax plasmids carrying *oriT* regions of other conjugative plasmids.

4.4. Determination of donation vs conduction as the mode of mobilization

The inability to demonstrate *in vitro* relaxation with pCU1 derivatives prevented the use of such techniques to confirm donation and localize the sequence carrying *nic*. However, on the assumption that the negative results obtained with the relaxation assay could be due to a lack of sensitivity of the *in vitro* assay system, attention was directed towards *in vivo* evidence of donation and demonstration of a transfer related *in vivo* nicking event.

4.4.1. Replica plating transconjugants to determine co-transfer of conjugative and non-conjugative plasmids

In an effort to differentiate between donation and conduction, transconjugants from matings involving pCU101 and each of the *oriT* derivatives were replica plated

from the plates selecting for only the *oriT* derivative onto plates selecting for the conjugative plasmid. Identification of transconjugants containing the non-conjugative plasmid marker without the conjugative plasmid marker would indicate that either the *oriT* derivative was transferred on its own or that both the *oriT* derivative and the helper plasmid were transferred but that once transferred, the conjugative plasmid was lost under the non-selective conditions.

pCU101 has been shown to be unstable in *E. coli* when antibiotic selection is released (personal observation and personal communication from Paul Hengen, also working on pCU1 in this laboratory). As a result, the transconjugants plated on media selecting for only the *oriT* derivative lose pCU101 at a high frequency. This loss would not occur if pCU101 was integrated into the *oriT* plasmid for which antibiotic selection is maintained. As a result, in transconjugants receiving both pCU101 and the *oriT* derivative by donation (as separate elements), the pCU101 plasmid is subject to instability while in transconjugants receiving pCU101 and the *oriT* plasmid as a co-integrate via conduction, the pCU101 plasmid is stabilized by the selection for the *oriT* derivative to which it is covalently linked.

Upon examination of the replica plating results obtained in the pCU101 matings (see Table 4.1), the four derivatives, pBP1, pBE60, pSP12 and pSP19 which were inferred to be donated due to high mobilization frequencies produced transconjugants lacking the conjugative plasmid. The frequency with which the Cm^r (the pCU101 marker) was lost ranged from 13-75% of the transconjugants tested. Although pSP25 transconjugants were not tested, one would predict that the results would be comparable.

Within the group of 14 *oriT* derivatives inferred to be conducted due to low

mobilization frequencies, there was a clear division. As seen with the higher mobility *oriT* derivatives, both pBP11 and its sister derivative, pSP14 consistently produced transconjugants carrying only the non-conjugative marker without the conjugative helper plasmid marker. The occurrence of these Cm^r transconjugants was slightly less frequent (10-40%) than with the higher mobility derivatives. pSP20 also produced Cm^r transconjugants. However, the frequency was significantly lower than seen with pBP11 and pSP14 (5%). The remaining 11 derivatives consistently produced transconjugants carrying both markers. Insufficient transconjugants were obtained from the matings involving pSP16 to allow proper assessment.

Transconjugants from matings with pCU1 and pCU401 as the helper plasmids were also replica plated to determine the frequency with which the conjugative plasmid's marker was lost. These tests demonstrated that transconjugants lacking the conjugative plasmid pCU1 were significantly less common with the derivatives inferred to be donated (pBP1, pBE60, pSP12, pSP19). They did not arise with the pBP11 or pSP14 or the other low mobility *oriT* derivatives. In the cases where pCU401 was used in place of pCU1, pSP14 matings did produce transconjugants without the helper plasmid while pSP13 and pBS47 did not. Whether this can also be attributed to instability of the helper plasmid is questionable as there is no information available on the stability of pCU401.

Taken together, the results presented in this section suggest that the lack of pCU101 in the transconjugants is not due to the lack of co-transfer but rather to the instability of the helper plasmid after transfer. The variation in the stability of pCU101 in the pBP11, pSP14 and pSP20 transconjugants as compared to those containing the other low mobility *oriT* derivatives, leaves open the possibility that

these derivatives may have been donated at a low frequency instead of, or perhaps in addition to being conducted.

4.4.2. Eckhardt gel electrophoresis of transconjugants

Eckhardt gel electrophoresis provides a quick method of visualizing the plasmid(s) within a cell (Eckhardt, 1978). The cells are lysed directly in the gel, and each plasmid in the cell can be seen as a unique band after electrophoresis. The location of the band on the gel is determined by the size of the plasmid. In the case of the donor cells used for the mobilization experiments outlined above, the large conjugative plasmid is seen close to the wells of the gel while the smaller *oriT* derivatives can be seen at the bottom of the gels. By comparing transconjugant cells with donor cells, it is possible to determine whether the conjugative and non-conjugative plasmids are separate or co-integrated and whether the size of either has been altered. The ability to discern alterations of the plasmids' size as the result of co-integration and resolution depends primarily upon the size of the plasmid and the extent of the alteration. Increases and deletions as small as 500 base pairs can be seen when the original size of the plasmid is less than 10 Kb. However, in the case of larger plasmids such as conjugative plasmids, in order to be visible on the Eckhardt gel, the increase or deletion must be much more significant.

Eckhardt gel electrophoresis was performed on transconjugants from matings between C600Nal and HB101 derivatives carrying pCU101 and one of the *oriT* derivatives to visually determine whether co-integrate structures formed between pCU101 and the *oriT* derivative during conjugal transfer. Representatives of these

Figure 4.16a - 4.16c. Eckhardt gel electrophoresis of transconjugants resulting from matings between C600NaI and a) HB101/pCU101/pBE60 b) HB101/pCU101/pMK2004, and c) HB101/pCU101/pBP11. Lane marked D in each gel represents the HB101 donor strain in each mating, the remaining lanes are individual transconjugant isolates. In the gel photographs, arrows indicate DNA bands corresponding to chromosomal DNA (CH), *oriT* derivatives (C), altered *oriT* derivatives (AO), pCU101 (101), or putative co-integrates (CO) of the two plasmids as well as dimers of either plasmid or the putative co-integrate (O^d , 101^d , CO^d). The resistance or sensitivity to chloramphenicol (Cm^r/Cm^s), the presence or absence of pCU101, the *oriT* plasmid or an altered form of the *oriT* plasmid and the putative co-integrate are summarized in the table below; dimers of the various plasmids are not indicated in the table.

	a									b									c												
	HB101/pCU101/pBE60									HB101/pCU101/pMK2004									HB101/pCU101/pBP11												
Transconjugant/Donor	1	2	3	4	5	D	6	7	8	9	1	2	3	4	D	5	6	7	8	9	1	2	3	4	5	D	6	7	8	9	
Cm ^r /Cm ^s	r	s	s	r	r	r	r	s	s	s	r	r	r	r	r	r	r	r	r	r	r	r	r	r	s	r	r	r	r	r	
putative cointegrate	-	-	-	-	-	-	-	-	-	-	+	+	+	+	-	+	+	+	+	+	-	-	-	-	-	-	-	-	-	+	-
pCU101	+	-	-	+	+	+	+	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	+	-	-	+	+	+	-	-	-
altered <i>oriT</i> derivative	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-
<i>oriT</i> derivative	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	-	-	-	-	-	-	+	-	+	+	+	+	+	+	+	+

c

b

a

gels can be seen in Figures 16a-c. Unless otherwise stated, the transconjugants were tested within 48 hours of the mating. Transconjugant isolates were taken from the NalKm plates and hence grown overnight in LB+Km. The lack of Cm selection allowed for the possible loss of pCU101.

Figure 16a demonstrates the plasmid content of nine randomly selected transconjugants from a mating between C600Nal and HB101/pCU101/pBE60. Upon comparison of the plasmid DNA present in the donor cell (lane 5) with that in the nine transconjugants (lanes 1-4 and 6-10) one can see that all nine transconjugants contain a DNA band which co-migrated with the pBE60 DNA (lower band) of the donor. Conversely, only three of the transconjugants contained a band co-migrating with the pCU101 DNA (upper band) of the donor. The presence or absence of the pCU101 band in the transconjugants corresponds well to the resistance or sensitivity of the transconjugant. Comparable results were obtained with randomly selected transconjugants from matings involving donors carrying pCU101/pBP1, pCU101/pSP12 and pCU101/pSP19 and support the conclusion that these four *oriT* derivatives are mobilized by donation as elements separate from the conjugative plasmid. Although Eckhardt gel electrophoresis was not conducted on transconjugants from matings involving pSP25, there is no reason to expect conflicting results with this *oriT* derivative.

Similar Eckhardt gel analysis conducted on transconjugants from matings between C600Nal and HB101/pCU101/pMK2004 demonstrated quite a different electrophoresis profile (Figure 16b). pMK2004 does not encode its own *oriT* and carries no pCU1-derived sequences. Hence the rare conjugal transfer seen with this plasmid must occur by conduction rather than donation. As can be seen in

Figure 16b, none of the transconjugants (lanes 1-4 and 6-10) demonstrate a band co-migrating with the pMK2004 DNA of the donor (lower band, lane 5) but all show a band which migrated more slowly than the pCU101 DNA of the donor (upper band, lane 5). Co-inheritance of the Km^r and Cm^r markers of the parent plasmids in all of these rare transconjugants (see Table 4.1) combined with presence of a plasmid larger than pCU101 but neither parent plasmid in the transconjugant indicates that pMK2004 was conducted. Similar electrophoresis results (data not shown) were obtained with *oriT* derivatives pBE42, pSP13, pSP15, pSP22, pSP23 which also do not yield Cm^r Km^r transconjugants (Table 4.1).

Eckhardt gel electrophoresis of transconjugants from matings involving two other low mobility *oriT* derivatives pBP11 and pSP14, produced results comparable to those obtain with the high mobility derivatives. All of the transconjugants tested contained an *oriT* plasmid separate from the pCU101 plasmid (Figure 4.16c lanes 1-5 and 7-10). In the majority of the transconjugants tested (18 of 27 pBP11 and 10 of 14 pSP14), the *oriT* plasmid co-migrated with the *oriT* plasmid in the donor (see Figure 15c, lower band, lanes 6). This suggests that in the majority of cases, these two plasmids are donated although at a much lower frequency than the derivatives illustrated in Figure 16a. Thus, regions present in derivatives such as pBP1 and pBE60 but absent in pBP11 and pSP14 must contribute to the efficiency of donation.

In the remaining pBP11 and pSP14 transconjugants, the *oriT* plasmid demonstrated an alteration in size as seen by an alteration in mobility on the Eckhardt gels (Figure 4.16c lanes 1 and 3). The alterations undergone by these rare pBP11 and pSP14 derivatives can be grouped into three categories. Two of the nine altered pBP11 derivatives and one of the four altered pSP14 derivatives

demonstrated a large increase in size (Figure 4.16c, lane 1), while two altered pBP11 and three altered pSP14 derivatives demonstrated smaller size increases (Figure 4.16c, lane 3). The last group consisted of five altered pBP11 derivatives showing a decrease in size (not shown). Each of the three alterations are believed to have resulted from co-integration with pCU101 followed by resolution in the recipient resulting in either an insertion of DNA (causing an increase in the size of the *oriT* derivative) or a deletion of DNA (causing an decrease in plasmid size). Restriction analysis of these altered *oriT* derivatives is described below (Section 4.4.3).

Like pBP11 and pSP14, pSP20 also produced transconjugants with a band co-migrating with the *oriT* derivative DNA in the donor. However, unlike pBP11 and pSP14, this was observed in only 4 of the 28 transconjugants tested (14%). In the remaining twenty-four transconjugants, a putative co-integrate was observed. In one of the four transconjugants showing a separate *oriT* plasmid, the migration distance differed from that of the pSP20 DNA in the donor. Similarly, one of the thirty five pSP21 transconjugants demonstrated a DNA band separate from pCU101 but it did not co-migrate with the pSP21 band in the donor. Restriction analysis of these derivatives is described in Section 4.4.4.

4.4.3. Restriction digest analysis of pBP11 and pSP14 derivative DNA altered during conjugal transfer.

Restriction analysis of the altered pBP11 and pSP14 derivatives indicated that within the accuracy of restriction mapping, the smaller 'insertion' derivatives of both plasmids carried identical 400 bp inserts. The two derivatives of each plasmid were isolated from separate matings. The two pBP11 and single pSP14 derivatives

showing larger insertions also carried apparently identical inserts of 5 Kb. Again the two pBP11 derivatives were isolated from separate matings. The apparent deletion derivatives of pBP11 were actually a combination of insert and deletion as will be detailed below. All five of the deletion derivatives were isolated from the same mating involving pBP11, no deletion derivatives of pSP14 were observed.

For all three types of alteration, restriction analysis demonstrated that the alteration occurred within the *Bam*HI/*Hind*III fragment carrying the pCU1 *ori*T DNA (Figure 4.17). Digestion with *Pst*I, *Hind*III, and *Hpa*I indicated that the derivatives with the 5 Kb insertion and the deletion derivatives contained DNA with *Hpa*I, *Pst*I and *Hind*III sites in the same position found in IS50 DNA suggesting that at least part of the inserted DNA consisted of IS50 DNA from the Tn5 in pCU101. In addition to IS50 DNA, the 5 Kb insert derivatives also contained most of the vector pACYC184 DNA from pCU101. Presence of pACYC184 DNA in the 5 Kb insert explains why the these isolates were resistant to chloramphenicol despite the lack of pCU101 DNA (see Figure 4.16c). In the altered pBP11 derivative with the apparent deletion, the IS50 like DNA was accompanied by a deletion of DNA within the region between the two *Hind*III sites of the pMK2004 derived DNA in pBP11/pSP14. This can be seen in by the loss of the 2.67 Kb *Hind*III fragment in this derivative (Figure 4.17 lane 5) as compared to pBP11 as well as the other two types of alterations (Figure 4.17 lanes 2, 3, 4 and 6).

Upon first consideration of these results, one might suspect that a co-integrate between the *ori*T derivative and pCU101 may have resulted from the transposition of the IS50 from the latter. Although this is a valid hypothesis, there

Figure 4.17. *Bam*HI+*Hind*III restriction digest of pBP11 derivatives altered during mobilization by pCU101. *Bam*HI-*Hind*III fragment carrying *oriT* region in pBP11 is the 1.33 Kb fragment indicated by arrow. Lane 1 is the 1 Kb DNA fragment ladder (BRL). Large (>12 Kb) fragment found in lanes 3, 5 and 6 is from pCU101 which was also present in the transconjugants used to extract CsCl plasmid DNA. Note, this fragment is not present in either lane 2 (pBP11 DNA alone) or lane 4 (the 5 Kb insert transconjugant). Although the 5 Kb insert transconjugants did not carry pCU101 it does show a 3.97 Kb fragment comparable to the *Hind*III fragment of pACYC184 from pCU101 found in lanes containing the >12 Kb fragment. This is presumably due to the insertion of this fragment in the altered derivative.

Lane 2 pBP11	Lane 3 unaltered derivative	Lane 4 5 Kb insert derivative	Lane 5 deletion derivative	Lane 6 400 bp insert derivative
3.83 ^b	>12 ^a 3.97 ^a	>12 ^a 3.97 3.48 ^c	>12 ^a 3.97 ^a	3.97 ^a
2.67	2.67	2.67 2.40	2.40	2.67
1.33	1.33		1.40	1.75
0.75	0.75	0.75	0.75	0.75

^a fragment from pCU101 also present in the cell

^b fragment due to incomplete digest ($2.57 + 1.33 = 3.9$)

^c fragment due to incomplete digest ($2.67 + 0.75 = 3.42$)

are several points to discount it.

First, pCU101 does not carry the entire IS50 element. It lacks the 320 bp *BglII-HindIII* fragment carrying the inner sequence shown to be necessary for transposition (Phadnis and Berg, 1987). Secondly, despite the fact that the altered pBP11 and pSP14 *BamHI/HindIII* fragment carries a known hot spot for Tn5 insertion within the tetracycline promoter (Berg *et al.*, 1983) restriction analysis showed no variation in the 0.9 Kb *PstI/EcoRV* fragment known to carry the hot spot. Thirdly, the 400 bp insertion derivatives did not possess additional *HpaI*, *PstI* or *HindIII* sites found in the IS50 element. Finally, the lack of similar results with pSP13, pSP15, pSP17 or pBS47 which carry all of the 1.4 Kb *BamHI/HindIII* fragment save the internal *SspI* fragment strongly suggests that the pCU1 *oriT* region is involved in the co-integration of pCU101 and pBP11 or pSP14.

The apparent involvement of the *oriT* in the alteration process suggests that the co-integration of the *oriT* plasmid with pCU101 may have occurred by site-specific recombination at the *nic* sites of the two plasmids. By analogy with the transfer systems of other Gram negative eubacteria, it is reasonable to assume that transfer is initiated within the minimal *oriT* region by strand-specific nicking at the site called *nic*. This produces free ends of DNA which would be available for site specific recombination to occur forming a co-integrate between the conjugative and non-conjugative plasmids at *nic*. As indicated in the Literature Review, co-integration via CSSR at the *oriT* site has been observed with both F and ColE1 plasmids (Warren and Clark, 1980, Horowitz and Deonier, 1985, Thompson *et al.*, 1984). Resolution could then occur in the transconjugant by either a second site-specific event or by homologous recombination as the recipient used in all of the matings

was *RecA*⁺. Isolation of identical inserts in both pBP11 and pSP14 as well as in each plasmid in two separate matings suggests that the alterations resulted from different resolution events of the same co-integrate structure. Figure 4.18a illustrates a possible co-integrate structure. The three different resolution events which would lead to the three types of alterations observed is outlined in Figure 4.18b-e. Similar structures can be proposed for altered pSP14 derivatives.

There are several sequences of homology between pCU101 and *oriT* derivatives pBP11 and pSP14. The most obvious region of homology is the pCU1 *oriT* sequences carried by each. Because these homologous sequences are in the same orientation (directly repeated), recombination between them would result in a deletion of the intervening DNA and resolve the co-integrate without apparent alteration to either original plasmid (see Figure 4.18a). In actuality, the resultant plasmids would have exchanged the DNA region between *nic* and the site of recombination; however, since the DNA should be identical, this event could not be detected. This possibility raises the question of whether the pBP11 and pSP14 transconjugants carrying apparently unaltered *oriT* plasmids, actually received such by low efficiency donation, or whether they were really conducted via site-specific recombination at *nic* and resolved by homologous recombination between *oriT* sequences.

A second region of homology carried by pCU101, pSP14 and pBP11 is the *Hind*III-*Bam*HI fragment carrying the promoter proximal end of the same tetracycline gene (originally from the pACYC184 vector DNA of pCU101 or the pMK2004 vector DNA of pBP11 or pSP14). In all three plasmids the tetracycline gene has been inactivated and in the case of the *oriT* derivatives, the *Bam*HI site was lost in the

Figure 4.18. Proposed co-integrate structure between pCU101 and pBP11 leading to the formation of the three altered pBP11 derivatives found in transconjugants from matings C600Nal and HB101/pCU101/pBP11. The DNA has been shown as a linear element, however, in all cases, the ends of each are linked to form a circular molecule

a) proposed co-integrate structure formed via CSSR within *oriT* sequences of pCU101 and pBP11. pCU101 portion of the co-integrate is indicated by the lower double line (only the portion of pCU101 involved in the recombination events have been shown) and pBP11 DNA by the upper double line, the broken vertical line indicating the joint between the two plasmids. Hatched region (▨) indicates *oriT* region, stipled region (▤) indicates the tetracycline promoter region, the solid region (■) indicates the Tn5 terminus and the *oriV* sites of each plasmid are indicated by V. Restriction site symbols are listed in Figure 4.3, Cm^r, chloramphenicol resistance, Km^r, kanamycin resistance, Ap^r, ampicillin resistance and Tra, transfer region. Single lines below the double lines indicate the *Bam*HI-*Hind*III restriction fragment with sizes in Kb as determined from Figure 4.17.

b) unaltered pBP11 resulting from homologous recombination between *oriT* sequences found in both pBP11 and pCU101

c) 5 Kb insert derivative resulting from homologous recombination between the two tetracycline promoter regions of the proposed co-integrate

d) 'deletion' derivative resulting from a second site-specific recombination event between the two *oriV* sites of the proposed co-integrate

e) 400 bp insert derivative resulting from homologous recombination between Tn5 terminus sequences in the proposed co-integrate.

construction of pSP1. Again these homologous regions are in the same orientation (directly repeated) and as a result, recombination between the two would result in the deletion of the intervening DNA (in this case the region carrying the *tra* region of pCU101). As illustrated in Figure 4.18c, the sequence between the homologous tetracycline promoter and *nic* of pBP11 or pSP14 would be replaced by the same region in pCU101. This region in pCU101 includes the larger *nic*-IS50 pCU1 (since the Tn5 sequence is 400 bp further from the end of *tra* in pCU101 than in pVT30) sequence as well as all of the IS50 element DNA found in pCU101 (up to the *HindIII* site) and all of the pACYC184 vector DNA found in pCU101. The presence of the pACYC184 DNA in the insertion derivative explains why the transconjugants did not show a pCU101 band on the Eckhardt gels but were still resistant to chloramphenicol (see Figure 4.15c).

The insertion/deletion derivative could be derived from a site-specific recombination event between the two *oriV* sites on the co-integrate (Figure 4.18d). As with *oriT* sequences, *oriV* sequences also undergo site-specific nicking which provide free DNA ends capable of undergoing recombination with other free DNA ends. Such CSSR between *oriV* sequences has been observed with the F plasmid *oriV* (O'Connor and Malamy, 1984, Kilbane and Malamy, 1980). Joining of the free DNA ends within the two *oriV* sequences present on the proposed co-integrate would produce the smaller alteration derivative which contains IS50 DNA from pCU101 and the pCU101-pBP11 *oriT* junction. Although this type of derivative was isolated from as many transconjugants as the two other types combined, the fact that all five isolates were isolated from the same mating involving pBP11 and no deletion derivatives were isolated from the two matings involving pSP14, suggests that it may

not be as common an event as the numbers would suggest.

The 400 bp insertion derivative (Figure 4.18e) can be pictured to have derived from a homologous cross over between the IS50 sequences found in pBP11, pSP14 and pCU101. The former two plasmids carry a 185 bp IS50 sequence representing the terminal *HpaI* fragment while the latter carries the same terminus plus an additional one Kb to the *HindIII* site. As seen with the previous regions of homology, these sequences are in the same orientation in the co-integrate such that homologous recombination between the two results in the deletion of the intervening DNA. The result of the cross over would replace the pBP11 or pSP14 DNA extending from the *nic* to the IS50 DNA with the same region of the pCU101 plasmid. As demonstrated in section 4.2.1, the Tn5 insert (and hence IS50) found in pCU29, from which pCU101 was constructed is approximately 0.4 Kb further from the *oriT* region than the Tn5 insert in pVT30 from which pSP1 and hence pBP11 was constructed. The exchange of DNA between the two IS50 sequences would result in the replacement of the pBP11 IS50-*nic* region with a homologous but 400 bp larger region from pCU101. This is the 400 bp insertion seen in these derivatives.

4.4.4. Restriction analysis of pSP20 and pSP21 derivatives altered during conjugation.

Restriction analysis of the altered pSP21 plasmid demonstrated that the *BamHI-HindIII* fragment not containing the *oriT* region had increased in size by approximately 1.3 Kb. These results suggest that the region between the kanamycin gene and the *oriT* region was involved in the alteration and that the putative *nic* site was not involved.

In the altered pSP20 isolate, one of the *SspI* sites within the *oriT* region and the *Bam*HI site at the end of the *oriT* region were lost; however, the resultant derivative could not be explained by site-specific recombination between *nic* sites as proposed for pBP11 or pSP14. All possible combinations of junctions between *oriT* and *oriV* sites to form a co-integrate structure between the two plasmids in all possible orientations were analyzed for possible recombination events which would result in the observed restriction pattern but no plausible intermediate could be identified. It is therefore concluded that this altered pSP20 derivative represents spontaneous deletion of a larger structure, possibly a pCU101-*oriT* derivative co-integrate; however, the structure of such a co-integrate is unknown.

One point of interest lies in the fact that pSP20 lacks both the Tn5 and tetracycline promoter sequences believed to be involved in the resolution event by homologous recombination which resulted in the altered pBP11 and pSP14 derivatives (see Figure 4.10). This may explain why there were no isolates with alterations similar to those found in pBP11 and pSP14 despite the presence of separate pSP20 elements in the transconjugants consistent with donation. As with pBP11 and pSP14, resolution of a co-integrate linked at *nic* sites could occur between the two homologous *oriT* sequences of the co-integrate to produce unaltered isolated resolution products.

4.5. Identification of plasmid specific *trans* acting *tra* functions

4.5.1. Specificity for *mob* functions among N type transfer systems.

As previously indicated, plasmid specific *mob* functions have been

demonstrated for members of both the F and P incompatibility groups (Willett and Maule, 1985; Yakobson and Guiney, 1983). In both cases, the functions encoding this specificity were found to include those involved in the relaxation of the plasmid DNA due to single-stranded nicking. An effort was made to determine whether the IncN plasmids also demonstrate plasmid specificity with respect to *mob* functions. If such a specificity could be identified their relationship to relaxation functions could be investigated.

Because six of the eight *tra* complementation regions of pCU1 are involved in pili production (Thatte *et al.*, 1985₁), it was suspected that the two remaining complementation groups carried on the region extending from the *Sma*I site at approximately 2.7 Kb on the pCU1 map to the end of the *tra* region would most likely be responsible for conjugal DNA metabolism. This region has been expanded in Figure 4.19.

Eight conjugative plasmids encoding an N type *tra* systems were tested for their ability to mobilize three non-conjugative derivatives, pCU57 and pCU56 (or pCU404 carrying the same pCU1 region in a different vector) and pBP1. pCU57 contains pCU1 DNA extending from 0 Kb site of pCU1 clockwise to 9.55 Kb on the pCU1 map (to the *Tn5* insert of pCU29) map while pCU56 or pCU404 carries the region from the *Bgl*II site at approximately 7 Kb on the pCU1 map clockwise to the *Bgl*II site past the end of *tra* at approximately 11.5 Kb and pBP1 carries only the *oriT* region (Figure 4.19). It was believed that if pCU1 specific *mob* functions existed they would most likely be encoded by pCU57 but possibly not by pCU56 or pCU404 and almost certainly not by pBP1. A variation in mobilization frequencies of the three plasmids by different IncN conjugative plasmids would indicate specificity in *mob*

functions similar to that found in the P and F *tra* systems.

As seen in Table 4.3, all of the self-transmissible, IncN plasmids tested were able to mobilize pCU57, pCU56 and pBP1 at comparable frequencies except for pSP274 and pSP545. These two plasmids are derived from a collection of plasmids call the pMUR plasmids isolated in the pre-antibiotic era (Datta and Hughes, 1983, Hughes and Datta, 1983). These plasmids, while able to mobilize pCU57 as efficiently as the other IncN conjugative plasmids (10^{-1}), demonstrated a reduced ability to mobilize pCU56 (10^{-4}) and negligible ability to mobilize pBP1 ($<10^{-6}$). It is evident from these results that as the amount of pCU1 DNA extending from the *oriT* end of *tra* decreased in size on the non-conjugative test plasmid, the ability of the pMUR plasmids pSP274 and pSP545, to mobilize the plasmid also decreased. These results indicate the existence of plasmid specificity among plasmids encoding N transfer systems which is not associated with pili functions.

4.3.2. Localizing the pCU1 specific *mob* functions

In order to characterize the pCU1 region encoding the plasmid specific *mob* functions it was necessary to construct additional derivatives carrying portions of the region from the 0 co-ordinate of pCU1 to the end of *tra*. Three approaches were taken: subcloning known restriction fragments, deletions using known restriction sites within the region and Bal31 deletion mutagenesis. These procedures are outlined below and the resultant constructs are presented in the lower portion of Figure 4.19.

^a plasmids are in HB101

^b number of transconjugants receiving the conjugative plasmid or non conjugative marker per donor.

^c number of tranconjugants receiving the non-conjugative plasmid per transconjugant receiving the conjugative plasmid.

^d pSP274 and pSP545 are Tn10 insertion derivatives of pMUR274 and pMUR545 respectively constructed as outlined in Methods and Materials section 3.12. Both are Tra⁺.

Table 4.3. Mobilization of pBP1, pCU57 and pCU56/pCU404 by IncN plasmids

Plasmids ^a transfer	Conjugative transfer frequency ^b	Non-conjugative transfer frequency ^b	Mobilization frequency ^c
pCU1/pBP1	7.8×10^0	4.2×10^0	5.4×10^{-1}
pCU1/pCU56	9.2×10^0	5.0×10^0	5.4×10^{-1}
pCU1/pCU57	3.5×10^0	1.8×10^0	5.1×10^{-1}
R113/pBP1	2.7×10^0	1.9×10^0	7.0×10^{-1}
R113/pCU56	8.4×10^0	1.2×10^0	1.4×10^{-1}
R113/pCU57	7.1×10^{-1}	8.1×10^{-1}	1.1×10^0
pTM4245/pBP1	5.3×10^0	4.4×10^0	8.3×10^{-1}
pTM4245/pCU56	3.6×10^0	1.1×10^0	3.1×10^{-1}
pTM4245/pCU57	6.2×10^{-2}	5.8×10^{-2}	9.4×10^{-1}
R199/pBP1	1.2×10^0	1.8×10^0	1.5×10^0
R199/pCU56	1.2×10^{-1}	1.0×10^0	8.3×10^0
R199/pCU57	2.0×10^{-1}	1.2×10^0	6.0×10^0
R45/pBP1	5.5×10^0	8.3×10^0	1.5×10^0
R45/pCU56	7.5×10^0	3.5×10^0	4.7×10^{-1}
R45/pCU57	8.0×10^{-1}	1.8×10^0	2.3×10^0
R46/pCU56	1.8×10^0	4.3×10^0	2.4×10^0
R46/pCU57	5.4×10^0	5.6×10^0	1.0×10^0
pSP274 ^d /pBP1	1.6×10^1	1.4×10^{-5}	8.8×10^{-7}
pSP274/pCU404	5.7×10^{-1}	6.8×10^{-5}	1.2×10^{-4}
pSP274/pCU57	1.1×10^1	2.8×10^0	2.5×10^{-1}
pSP545 ^d /pBP1	1.6×10^0	$< 1.0 \times 10^{-7}$	6.3×10^{-8}
pSP545/pCU404	4.0×10^0	7.0×10^{-4}	1.8×10^{-4}
pSP545/pCU57	6.9×10^0	8.7×10^{-1}	1.3×10^{-1}

a) Construction of deletions into *mob*

Reduced mobility of pCU56 by the pMUR plasmids combined with the knowledge that pili functions are encoded by the region counterclockwise from the 2.7 Kb co-ordinate suggested that the region carried on the *Bgl*II fragment extending from 3.3-7 Kb on the pCU1 map (see Figure 4.1), may encode at least some of the plasmid-specific Mob functions. A composite derivative, pBGL, was constructed by subcloning this *Bgl*II fragment into the *oriT* derivative pBP1. pBP1 plasmid DNA was linearized at the unique *Bam*HI site (at the end of the pCU1 derived DNA) and ligated to pCU57 DNA digested with *Bgl*II. Plasmid DNA isolated by the rapid boiling method was digested with both *Kpn*I and *Pvu*I to identify those clones carrying the desired *Bgl*II fragment. The orientation of the *Bgl*II fragment in pBGL is such that the *Bam*HI site of pBP1 representing the 8.5 Kb co-ordinate of pCU1 is ligated to the 3.3 Kb end of the *Bgl*II fragment.

Rather than attempting to construct a derivative carrying the region from *oriT* counterclockwise to the *Bgl*II site at 3.3 Kb on the pCU1 map (difficult due to the intervening *Bgl*II site at 7.0 Kb), the pBGL derivative was combined in the same cell with pCU56 to produce a strain carrying the complete region on two separate, compatible plasmids which could complement each other in *trans*.

Finally, pBGL was used to produce Bal31 deletions into the 3.3-7 Kb *Bgl*II fragment counterclockwise from 7 Kb. pBGL DNA was linearized at the unique *Xho*I site and digested with Bal31 at 25°C for 2-10 min. The ends were filled in and *Bam*HI linkers added. Following ligation the DNA was transformed into HB101 and transformants screened by *Kpn*I/*Bam*HI restriction digestion of mini-prep DNA. The

Figure 4.19. The *mob* region of pCU1 and pKM101. The upper portion of this figure is a comparison of pCU1 and pKM101 *mob* regions. Double lines represent pKM101 and pCU1 regions extending from the *Sal*I sites located at co-ordinates 10.8 and 0 Kb respectively. Complementation regions are indicated within the boxes and the *oriT* region is indicated below. *nuc* and *fip* are regions determining a nuclease and fertility inhibition of P group plasmids respectively. Information for pKM101 is from Winans and Walker, 1985₂. Information for pCU1 is taken from Thatte *et al.*, 1985, however, more recent sequence analysis of the pCU1 region extending from the *Sma*I site to the end of *tra* has demonstrated that the actual location of restriction sites is closer to that found in pKM101 than initially reported for pCU1. The position of the restriction sites reflects this new information. Broken lines between the two plasmids indicate comparable restriction sites. See Figure 4.2 for restriction site designations. *Pvu*I, *Pvu*II. No information on *Pvu*I sites in pKM101 was available.

The lower portion of the figure, illustrates the pCU1 *mob* derivatives used in complementation tests with pSP274 and pSP545. pCU56, pCU57, pBP1 and pBGL are indicated along with deletion derivatives of pCU57, pCU109 and pBGL. The extent of the deletions into pCU57 were determined by *Bam*HI/*Bgl*II digests. Deletions into pBGL were determined by *Kpn*I/*Bam*HI digests. Below, the size of the fragment generated is compared to the original fragment.

<i>Bgl</i> II- <i>Bgl</i> II		<i>Kpn</i> I- <i>Bgl</i> II	
pCU57	3.5 Kb	pBGL	1.5 Kb
pCU57 Δ 14	3.4	pBGL Δ 31	1.4
pCU57 Δ 37	2.9	pBGL Δ 45	1.4
pCU57 Δ 18	2.6	pBGL Δ 15	1.2
pCU57 Δ 71	2.2	pBGL Δ 47	1.2
		pBGL Δ 5	1.1

four derivatives obtained are shown in Figure 4.19.

To obtain deletions from the 3.3 Kb end of the 3.3-7 Kb *Bgl*III fragment, pCU57 plasmid DNA linearized with *Sma*I restriction enzyme was subjected to *Bal*31 digestion for 5-20 min at 30°C. *Bam*HI linkers were added and the DNA recircularized and transformed into HB101. Plasmid DNA was extracted by the rapid boiling method from the transformants and digested in a *Bgl*III/*Bam*HI double digest in order to determine the extent of the deletions. Four derivatives spanned the region from the *Sma*I site to the *Pvu*I site at approximately 5 Kb on the pCU1 map (Figure 4.19).

Unfortunately, the presence of unique restriction sites within the region extending from the zero co-ordinate on the pCU1 map to the end of *tra* is limited to one *Kpn*I and one *Pvu*I site (Figure 4.19). pCU109 DNA, digested to completion with either *Kpn*I or *Pvu*I restriction endonuclease, were separately religated and transformed into HB101. Plasmid DNA was isolated by the rapid boiling method and digested with *Kpn*I or *Pvu*I to identify derivatives carrying the desired deletion.

b) Mobilization experiments on the *mob* deletion constructs

pSP274 and pSP545 were introduced into the deletion derivatives by mini-filter matings and transconjugants purified. The results of the mobilization experiments on these strains are shown in Table 4.4.

On comparing the mobilization frequency of pBP1 and pBGL (Table 4.4) it is apparent that the pCU1 region from 3.3-7 Kb subcloned on the *Bgl*III fragment does not significantly increase the mobility of the pBP1 derivative by the pMUR plasmids. However when pCU56 was added to the strain containing pBGL, mobilization of both

Table 4.4. Mobilization of pCU57, pCU56, pBP1, pBGL and their derivatives by pCU1 and the pMUR::Tn10 derivatives pSP274 and pSP545^a.

Plasmids ^b	Transfer frequency ^c		Mobilization frequency ^d	
	conjugative plasmid	non-conjugative plasmid 1	non-conjugative plasmid 2	non-conjugative plasmid 1 non-conjugative plasmid 2
pCU1/pBP1	7.8x10 ⁰	4.2x10 ⁰		5.4x10 ⁻¹
pCU1/pBGL	4.0x10 ⁰	4.3x10 ⁰		1.1x10 ⁰
pCU1/pCU56	9.2x10 ⁰	5.1x10 ⁰		5.5x10 ⁻¹
pCU1/pCU57	1.5x10 ⁰	5.8x10 ⁰		3.9x10 ⁰
pCU1/pCU57Δ14	6.0x10 ⁻¹	2.9x10 ⁻¹		4.8x10 ⁻¹
pCU1/pCU57Δ37	2.4x10 ⁰	1.6x10 ⁰		3.7x10 ⁻¹
pCU1/pCU57Δ18	7.9x10 ⁰	3.9x10 ⁰		4.9x10 ⁻¹
pCU1/pCU109ΔPv	5.6x10 ⁰	2.9x10 ⁰		5.2x10 ⁻¹
pCU1/pCU109ΔKp	5.7x10 ⁰	2.5x10 ⁰		4.4x10 ⁻¹
pSP274/pBP1	1.6x10 ¹	1.4x10 ⁻³		8.8x10 ⁻⁷
pSP274/pBGL	1.7x10 ⁰	9.3x10 ⁻⁷		5.5x10 ⁻⁷
pSP274/pCU56	2.1x10 ⁰	7.9x10 ⁻³		3.8x10 ⁻³
pSP274/pCU56/pBGL	6.4x10 ⁻¹	1.4x10 ⁻¹	2.3x10 ⁻¹	2.2x10 ⁻²
pSP274/pCU56/pBGLΔ45	2.5x10 ⁻¹	1.1x10 ⁻³	<1.4x10 ⁻³	4.4x10 ⁻⁵
pSP274/pCU56/pBGLΔ15	6.5x10 ⁰	2.6x10 ⁻⁴	<5.3x10 ⁻⁴	4.0x10 ⁻⁵
pSP274/pCU56/pBGLΔ47	4.9x10 ⁻¹	1.8x10 ⁻³	<4.1x10 ⁻³	3.7x10 ⁻⁵
pSP274/pCU56/pBGLΔ5	1.2x10 ¹	8.6x10 ⁻⁴	<3.1x10 ⁻³	7.2x10 ⁻⁵
				3.6x10 ⁻¹
				<5.6x10 ⁻⁵
				<8.2x10 ⁻⁵
				<8.4x10 ⁻⁵
				<2.6x10 ⁻⁵

pSP274/pCU57	5.7x10 ⁰	1.2x10 ⁰		2.1x10 ⁻¹	
pSP274/pCU57 Δ 14	2.1x10 ⁰	5.1x10 ⁻⁵		2.4x10 ⁻⁵	
pSP274/pCU57 Δ 37	2.7x10 ⁰	3.1x10 ⁻⁵		1.1x10 ⁻⁵	
pSP274/pCU57 Δ 18	5.5x10 ⁰	6.6x10 ⁻⁴		1.2x10 ⁻⁴	
pSP274/pCU57 Δ 71	3.1x10 ⁰	4.1x10 ⁻⁵		1.3x10 ⁻⁵	
pSP274/pCU109 Δ Pv	6.7x10 ⁰	1.7x10 ⁻⁶		2.5x10 ⁻⁷	
pSP274/pCU109 Δ Kp	4.5x10 ⁰	4.7x10 ⁻⁴		1.0x10 ⁻⁴	
pSP274/pCU57 Δ 14/pBGL Δ 5	6.5x10 ⁻¹	2.0x10 ⁻⁵	3.7x10 ⁻⁵	3.1x10 ⁻⁵	5.7x10 ⁻⁵
pSP274/pCU57 Δ 14/pBGL Δ 31	2.7x10 ⁰	3.1x10 ⁻²	3.4x10 ⁻⁴	1.1x10 ⁻⁴	1.3x10 ⁻⁴
pSP274/pCU109 Δ Kpn/pBGL Δ 5	5.3x10 ⁰	4.4x10 ⁻⁴	4.4x10 ⁻⁴	8.3x10 ⁻⁵	8.3x10 ⁻⁵
pSP274/pCU109 Δ Kpn/pBGL Δ 31	1.0x10 ⁰	4.7x10 ⁻⁵	2.3x10 ⁻⁴	4.7x10 ⁻⁵	2.3x10 ⁻⁴
pSP545/pBP1	1.6x10 ⁰	<1.0x10 ⁻⁷		<6.3x10 ⁻⁸	
pSP545/pBGL	2.0x10 ⁻¹	1.8x10 ⁻⁷		9.0x10 ⁻⁷	
pSP545/pCU56	5.8x10 ⁰	2.8x10 ⁻⁴		4.8x10 ⁻⁵	
pSP545/pCU56/pBGL	5.9x10 ⁰	9.8x10 ⁻²	1.1x10 ⁻¹	1.7x10 ⁻²	1.9x10 ⁻²
pSP545/pCU57	1.5x10 ⁰	3.4x10 ⁻¹		2.3x10 ⁻¹	
pSP545/pCU57 Δ 14	7.4x10 ⁰	6.5x10 ⁻⁵		8.8x10 ⁻⁶	
pSP545/pCU57 Δ 37	8.3x10 ⁻¹	2.5x10 ⁻⁶		3.0x10 ⁻⁶	
pSP545/pCU57 Δ 18	3.8x10 ⁰	1.1x10 ⁻⁴		2.9x10 ⁻⁵	
pSP545/pCU57 Δ 71	4.6x10 ⁰	9.8x10 ⁻⁵		2.1x10 ⁻⁵	
pSP545/pCU109 Δ Pv	8.6x10 ⁰	6.2x10 ⁻⁵		7.2x10 ⁻⁶	
pSP545/pCU109 Δ Kp	4.7x10 ⁰	1.2x10 ⁻⁴		2.6x10 ⁻⁵	

^a pSP274 and pSP545 are Tra⁺ Tn10 derivatives of pMUR274 and pMUR545 respectively.

^b plasmids were maintained in HB101.

^c number of transconjugants receiving plasmid antibiotic marker per donor.

^d number of transconjugant receiving non-conjugative plasmid marker per transconjugant receiving conjugative plasmid marker.

derivatives by the pMUR plasmids was completely restored. These results provide two important pieces of information. First, they demonstrate that the pCU1 specific mob functions are encoded on the region clockwise from the *Bgl*II site at 3.3 Kb on the pCU1 map (Figure 4.19). Second, the fact that complementation between the two plasmids in *trans* allowed mobilization indicates that this region contains at least two complementation regions, one on each side of the *Bgl*II site at 7 Kb on the pCU1 map. This agrees with the complementation map of pKM101.

The low mobilization frequencies for the pCU57 *Bal*31 deletion derivatives indicate that any loss of DNA clockwise from the 3.3 Kb *Bgl*II site (Figure 4.19) reduces the mobilization frequency by a factor of 5 to 6. Similar reductions in mobilization are noted with deletions extending into the other end of this *Bgl*II fragment as seen with the pBGL *Bal*31 deletion derivatives (Table 4.4).

Winans and Walker (1984) demonstrated that pKM101 encodes two complementation regions within the *Bgl*II fragment corresponding to the *Bgl*II fragment from 3.3-7 Kb on pCU1. Complementation tests between two different pCU57 deletion mutants and two pBP1-*Bgl*II deletion mutants were conducted to determine if this was also the case with pCU1. The mobilization results by the pMUR plasmid pSP274 are presented in Table 4.4 and indicate no evidence of complementation as seen by an increase in mobilization frequencies. These results may explain why the mobilization frequencies with the pBGL deletion derivatives were similar to those of the pCU57 deletion results.

4.5.3. Complementation of *Tra*⁻ pKM101::Tn5 insertion mutants by pMUR plasmids

As previously indicated, pKM101, a *Tra*⁺ deletion derivative of the IncN plasmid R46, possesses striking restriction pattern similarities to pCU1 within the *tra* region particularly in the pCU1 region extending from 0 Kb to the right hand end of *tra* (Figure 4.19). This study has shown that the sequence of the pCU1 *oriT* region is essentially the same as that determined by Coupland *et al.* (1987) for the *oriT* region of pKM101. These similarities between pCU1 and pKM101 has prompted the use of pKM101::Tn5 insertion mutants in the examination of *mob* function specificity among the IncN plasmids.

Figure 4.19 shows a map of the five complementation regions in the *oriT* proximal end of the *tra* region of pKM101 which are not involved in pili production. One is known to cause fertility inhibition of the IncP plasmids (*fip*). As with pCU1, *oriT* was located at the far right end of the *tra* region. To examine the plasmid specificity of each of the remaining four complementation regions, four pKM101 mutants carrying a Tn5 insertion in one of each of the four complementation regions were chosen for complementation tests with the pMUR derivatives pSP274 and pSP545.

pSP274 and pSP545 were separately introduced into each of the four pKM101::Tn5 mutants by mini filter matings. Large filter matings were then conducted on the purified transconjugants and the transfer frequency of the pKM101 derivative determined. The results are presented in Table 4.5.

Of the four pKM101 *tra* mutants tested, only the *traJ* mutant was

Table 4.5. Mobilization of pKM101::Tn5^a mutants by the pMUR Tn10 derivatives pSP274 and pSP545^b.

plasmid(s) ^c	pMUR transfer frequency ^d	pKM101 <i>tra</i> transfer frequency ^d	mob frequency ^e
pKM101 <i>traH1143</i>	N/A	1.4x10 ⁻⁶	N/A
pKM101 <i>traI1136</i>	N/A	1.9x10 ⁻⁶	N/A
pKM101 <i>traJ35</i>	N/A	2.8x10 ⁻⁸	N/A
pKM101 <i>traK1217</i>	N/A	<5.5x10 ⁻⁸	N/A
pSP274/pKM101 <i>traH1143</i>	1.4x10 ⁰	1.1x10 ⁻³	7.9x10 ⁻⁴
pSP274/pKM101 <i>traI1136</i>	8.2x10 ⁻¹	1.7x10 ⁻⁴	2.1x10 ⁻⁴
pSP274/pKM101 <i>traJ35</i>	7.8x10 ⁻³	2.2x10 ⁻¹	2.8x10 ⁻¹
pSP274/pKM101 <i>traK1217</i>	2.6x10 ⁰	5.0x10 ⁻⁶	1.9x10 ⁻⁶
pSP545/pKM101 <i>traH1143</i>	2.5x10 ⁻¹	7.5x10 ⁻⁵	3.0x10 ⁻⁴
pSP545/pKM101 <i>traI1136</i>	1.7x10 ⁻¹	1.2x10 ⁻⁴	7.0x10 ⁻⁴
pSP545/pKM101 <i>traJ35</i>	8.5x10 ⁻¹	1.7x10 ⁰	2.0x10 ⁰
pSP545/pKM101 <i>traK1217</i>	4.8x10 ⁰	>5.3x10 ⁻⁵	>1.1x10 ⁻⁵

^a Tn5 derivatives of pKM101 are described by Winans and Walker, 1985₂.

^b pSP274 and pSP545 are Tra⁺ Tn10 derivatives of pMUR274 and pMUR545 respectively. Construction of these derivatives is outlined in Methods and Materials section 3.12.

^c plasmids maintained in HB101.

^d number of transconjugants receiving plasmid marker per donor.

^e number of transconjugants receiving pKM101*tra* marker per transconjugant receiving pMUR plasmid.

complemented by the pMUR plasmids indicating that the pMUR plasmids produce a *traJ* like product.

Upon comparing the pKM101::Tn5 mobilization results (Table 4.5) with those obtained with the pCU1 *mob* derivatives (Table 4.4), we can see that inactivation or deletion of similar regions in the two plasmids have produced similar reductions in mobilization frequencies by the pMUR plasmids. The pKM101*traK* mutant has a mobilization frequency comparable to that of pBP1. Considering the functional DNA present on each derivative (Figure 4.19) one can conclude that the pKM101 *traK* equivalent is not carried by the pCU1 derivative pBP1. Similarly, the mobilization frequencies of pKM101*traH* and pKM101*traI* mutants are comparable to the pCU57 deletion derivatives and the pBGL deletion derivatives in the presence of pCU56.

4.5.4. Complementation of pKM101*traJ* mutations by conjugative plasmids encoding different *tra* systems

To investigate the possibility that the pKM101 *traJ* region encodes a transfer function common to transfer systems other than the N type transfer system, conjugative plasmids encoding an F type *tra* system (R386, R100-1), an I type (R64-11, R621a), an M type (R466b), a W type (S-a) and an X type (RA3) *tra* system were tested for complementation. The results of these mobilization experiments are presented in Table 4.6 and indicate that none of the transfer systems mobilize the pKM101 *traJ* mutation to any greater degree than the pKM101 *traH* mutation indicating that the *traJ* function is common only to the N type *tra* system.

Table 4.6. Mobilization of pKM101*traH1143* and pKM101*traJ35*^a by different transfer systems.

Plasmids ^b transfer	conjug. plasmid transfer frequency ^c	pKM101 transfer frequency ^c	mobilization frequency ^d
RA3/pKM101 <i>traH1143</i>	2.5×10^{-1}	2.5×10^{-4}	1.0×10^{-3}
RA3/pKM101 <i>traJ35</i>	6.6×10^{-2}	2.5×10^{-4}	3.8×10^{-3}
R100-1/pKM101 <i>traH1143</i>	6.4×10^{-1}	2.9×10^{-4}	4.5×10^{-4}
R100-1/pKM101 <i>traJ35</i>	5.0×10^{-1}	3.2×10^{-4}	6.4×10^{-4}
s-a/pKM101 <i>traH1143</i>	1.9×10^{-1}	1.5×10^{-5}	7.9×10^{-5}
s-a/pKM101 <i>traJ35</i>	1.8×10^{-1}	7.2×10^{-5}	4.0×10^{-4}
R621a/pKM101 <i>traH1143</i>	1.9×10^{-2}	2.6×10^{-7}	1.4×10^{-5}
R621a/pKM101 <i>traJ35</i>	1.5×10^{-2}	2.9×10^{-7}	1.9×10^{-5}
R64-11/pKM101 <i>traH1143</i>	1.5×10^{-1}	1.2×10^{-6}	8.0×10^{-6}
R64-11/pKM101 <i>traJ35</i>	6.8×10^{-2}	1.1×10^{-6}	1.6×10^{-5}
R386/pKM101 <i>traH1143</i>	1.1×10^{-1}	5.8×10^{-6}	5.2×10^{-5}
R386/pKM101 <i>traJ35</i>	1.2×10^{-1}	4.3×10^{-5}	3.6×10^{-4}
R446b/pKM101 <i>traH1143</i>	9.6×10^{-2}	2.7×10^{-6}	2.8×10^{-5}
R446b/pKM101 <i>traJ35</i>	2.2×10^0	2.4×10^{-5}	1.1×10^{-5}

^a pKM101::Tn5 insertion derivatives are described by Winans and Walker, 1985₂.

^b plasmids were maintained in HB101.

^c number of transconjugants receiving the plasmid antibiotic marker per donor.

^d number of transconjugants receiving the pKM101*tra* plasmid marker per transconjugants receiving the conjugative plasmid marker.

5. FURTHER DISCUSSION

Efficient mobilization of pSP3 by pCU1 indicates that the 2.15 Kb region of pCU1 DNA carried by this recombinant plasmid (from pVT30) encodes all of the *cis* acting functions required for conjugal transfer. This 2.15 Kb region corresponds to the sequence extending from the *Bgl*II site at 7.0 Kb on the pCU1 map to a Tn5 insert mapped at 9.15 Kb (1.35 Kb from the *Hpa*I site at 7.8 Kb). Furthermore, the fact that pVT30 is *Tra*⁺ leads to the conclusion that the *oriT* region represents the right hand limit of *tra* placing it at 9.15 rather than 8.2 as previously believed.

The two pSP3 *Bal*31 deletion derivatives, pBP1 and pBE60, carry deletions into opposite ends of the pCU1 DNA subcloned in pSP3. Both of these constructs are mobilized as efficiently as pSP3 further localizing the *cis* acting *tra* functions to the pCU1 region they share. Sequence analysis demonstrated that the region common to these two clones is 692 bp in size. The 23 bases at one end of the sequence correspond to the terminus of Tn5. This maps the 669 bp *oriT* region counterclockwise from 9.15 Kb to 3.48 Kb on the pCU1 map.

Statistical analysis of the mean mobilization frequencies from repeated matings involving pCU404, pSP3, pBE60 and pBP1 demonstrated that the small difference in frequency observed for pBP1 was significant at the 95% confidence level. Since pBP1 was constructed from pSP3 (and pCU404) and carries less pCU1 derived DNA, the fact that it is mobilized at a slightly higher frequency indicates that some factor(s) other than the DNA sequence per se present on the construct can affect the efficiency of mobilization. Such factors can include replicon type, antibiotic markers used for selection and conformation of the DNA. Since these four plasmids

were constructed from the same parental plasmid, they encode the same replicon and antibiotic markers. This suggests that secondary DNA structure may be responsible for altering the mobilization frequency. In essence, the DNA deleted from pSP3 in the construction of pBP1 may act to inhibit mobilization in pSP3. This inhibition may be the result of the secondary structure of neighbouring DNA sequences may limiting access of *mob* proteins to the *oriT* sequence.

As demonstrated by the mobilization frequencies of the other *oriT* derivatives constructed from pBP1 and pBE60, loss of DNA from either end of the 669 bp *oriT* region reduces mobilization frequency. In many instances it is possible to identify particular DNA sequence structures lost in the individual constructs which may be responsible for the reduced mobility.

The DNA deleted from pBP1 to construct pSP25 contains the thirteen 11 bp direct repeat sequences. This deletion resulted in a 2-3 fold decrease in mobility as compared to the mobilization frequencies of pCU404, pSP3 and pBE60. Statistical analysis of the results demonstrated that this reduction is significant at the 95% confidence level. However the fact that the difference is only slightly greater than the difference seen between pBP1 and pBE60 raises the question of whether the difference in mobilization frequency observed with pSP25 is due to the loss of the direct repeats or to placement of interfering DNA directly next to the *oriT* region. In essence the direct repeats may not be playing an active role in transfer as suggested by the small reduction in mobility observed with their loss, but rather a passive one by providing a 'buffer' sequence next to the *oriT* region allowing better access of the *mob* proteins. One argument for proposing a passive role for the direct repeats is the fact that although they represent a sequence one half the size

of the remaining portion of *oriT*, their loss has a only very small effect on mobility. Further study of the direct repeat sequences is required to fully understand their role in conjugative transfer and to determine the minimal *cis* acting *tra* region. Certainly however, it can be said that the 325 bp carried by pSP25 contains most if not all of the *cis* acting *tra* functions

Continued deletion of DNA from the left end of *oriT* results in further reductions in mobility. pSP12 demonstrates a 10 fold reduction in mobility relative to pBP1 and pBE60 strongly suggesting that the 10 bp inverted repeat located in the 33 bp sequence between the end of this derivative and that of pSP25 plays an active role in conjugative transfer. Deletion of an additional 36 bp to the *SnaBI* site in pSP19 results in an additional 10 fold decrease in mobility. In pSP19, one 6 bp inverted repeat, the dyad sequence and the first 14 bp direct repeat have all been deleted and hence it is difficult to attribute the decrease in transfer to one particular sequence feature. However, the reduction of transfer to background level with pSP20 corresponding to the loss of an additional 50 bp (to the *BspMI* site) and the second 14 bp direct repeat suggests that these direct repeats may play a role in transfer. As expected, pSP18, pSP11 and pBE42 are mobilized at background frequencies comparable to pSP20.

The low mobilization frequency of pBP11 and pSP14 demonstrates that the region carried to the right of the second *SspI* site is also necessary for efficient mobilization.

The alteration of pBP11 and pSP14 during mobilization by pCU101 provided evidence for an *in vivo* nicking event within the *oriT* region. This allows us to propose the placement of *nic* in the region to the left of the second *SspI* site. This

is contrary to the location proposed by Coupland *et al.* (1987) for pKM101 based on homology to pSC101. However, the homology observed between pSC101 and pKM101 (and pCU1) may reflect the presence of putative IHF binding sites rather than the location of *nic*.

Despite the lack of similar alterations with pSP20 (explained by the lack of homologous sequences found in pBP11 and pSP14), like pBP11 and pSP14 and the higher mobility derivatives (assumed to be donated), pSP20 did produce transconjugants lacking pCU101 (as seen by sensitivity to chloramphenicol) or containing the *oriT* plasmid separate from pCU101 (as seen by Eckhardt gel electrophoresis). These results suggest that pSP20 is also donated (albeit at a reduced frequency) and therefore possesses a *nic* site. This conclusion places *nic* in the 65 bp sequence between the *Bsp*MI and the second *Ssp*I site carried by both pBP11/pSP14 and pSP20. This eliminates both sequences showing homology to ColE1 and CloDF13 as possible locations of *nic*.

Within the 65 bp sequence proposed as the location of *nic* there is only one small 7 bp inverted repeat. This small inverted repeat does bear some consideration as a possible location of *nic* due to its close proximity to the 81 bp AT-rich region. The proposed *nic* sites of the F plasmid are also located directly next to the AT-rich region of the *oriT* of F. The lack of more significant inverted repeat sequences within this sequence may prove irrelevant as the proposed *nic* site of RP4 is not located in an inverted repeat.

As indicated above, the frequency of donation of pBP11, pSP14 and pSP20 is greatly reduced. However in the case of pBP11 and pSP14, all of the transconjugants tested by Eckhardt gel electrophoresis demonstrated evidence of

is contrary to the location proposed by Coupland *et al.* (1987) for pKM101 based on homology to pSC101. However, the homology observed between pSC101 and pKM101 (and pCU1) may reflect the presence of putative IHF binding sites rather than the location of *nic*.

Despite the lack of similar alterations with pSP20 (explained by the lack of homologous sequences found in pBP11 and pSP14), like pBP11 and pSP14 and the higher mobility derivatives (assumed to be donated), pSP20 did produce transconjugants lacking pCU101 (as seen by sensitivity to chloramphenicol) or containing the *oriT* plasmid separate from pCU101 (as seen by Eckhardt gel electrophoresis). These results suggest that pSP20 is also donated (albeit at a reduced frequency) and therefore possesses a *nic* site. This conclusion places *nic* in the 65 bp sequence between the *Bsp*MI and the second *Ssp*I site carried by both pBP11/pSP14 and pSP20. This eliminates both sequences showing homology to ColE1 and CloDF13 as possible locations of *nic*.

Within the 65 bp sequence proposed as the location of *nic* there is only one small 7 bp inverted repeat. This small inverted repeat does bear some consideration as a possible location of *nic* due to its close proximity to the 8 bp AT-rich region. The proposed *nic* sites of the F plasmid are also located directly next to the AT-rich region of the *oriT* of F. The lack of more significant inverted repeat sequences within this sequence may prove irrelevant as the proposed *nic* site of RP4 is not located in an inverted repeat.

As indicated above, the frequency of donation of pBP11, pSP14 and pSP20 is greatly reduced. However in the case of pBP11 and pSP14, all of the transconjugants tested by Eckhardt gel electrophoresis demonstrated evidence of

donation or *in vivo* nicking while pSP20, on the other hand, demonstrated that only 14 percent of the transconjugants tested received pSP20 by donation. These results suggest that the nicking process in pBP11 and pSP14 is more efficient than in pSP20. This in turn leads to the speculation that the region lacking in pSP20 may play a role in recognition of *oriT* by *mob* proteins characteristic of *bom* sequences. The lack of evidence for the donation of pSP21 despite the presence of the *Bsp*MI-*Ssp*I₂ fragment proposed to contain *nic* suggests that the *Sra*BI-*Bsp*MI fragment deleted in pSP21 may be critical to recognition. It is interesting to note that deletion of this small fragment removes both 14 bp direct repeat sequences and strengthens the evidence of their involvement in conjugation. Once *mob* proteins have been identified and purified, DNA protection experiments could be used to determine whether they do actually bind to the *oriT* region, and if so, which domain of the region is responsible for the binding.

Further support for the placement of *nic* within the *Bsp*MI-*Ssp*I₂ lies in the fact that pSP23 does not demonstrate evidence of donation. pSP23 has only the sequence to the left of the *Bsp*MI site. Accordingly no evidence of donation was obtained with other derivatives lacking this region proposed to encode *nic* (pSP22, pSP13, pSP15, pSP17, pSP11, pEB42). The results of the Eckhardt gel electrophoresis confirmed the formation of a co-integrate structure and hence conduction in these low mobility derivatives. Eventual resolution of these co-integrates however, did occur after extended incubation (greater than 48 hours, data not shown) into the original unaltered plasmids indicating that the integration and resolution events were the result of CSSR. Despite the fact that all of these co-integrates carried at least one set of homologous sequences (*oriT* sequences,

tetracycline gene and/or the terminus of Tn5) the lack of resolution products resulting from homologous recombination in the recipient suggests that in these cases the CSSR integration occurred at a site different than observed with pBP11 and pSP14 such that either the two plasmids were integrated such that the homologous sequences were not in the proper orientation to promote resolution or that the resolution products were not viable. A possible candidate for the location of the recombination is the *oriV* site of each plasmid.

The fact that all of the pBP11 and pSP14 transconjugants tested by Eckhardt gel electrophoresis demonstrated evidence of *in vivo* nicking suggests that the region to the right of the second *SspI* site is not involved in the nicking process. Other possible functions for this region which would have a significant effect on transfer is initiation of complementary strand synthesis. Several sequence features found in this region have been associated with DNA replication sequences in bacterial chromosome and plasmids and in bacteriophages. The first is the two large inverted repeat sequences. The secondary structure formed by these two inverted repeats may be recognized by a primase enzyme in the initial priming steps of DNA replication as seen in the single-stranded DNA phage G4. The second feature is the two sequences bearing homology to the IHF recognition sequence. Although IHF is believed to have a transcription regulatory role in the transfer of R100, the identification of the recognition sequence in many replicative origins suggests that it may also play a role in DNA synthesis. Investigation of the role played by IHF in the conjugative transfer of pCU1 has been complicated by the fact that pCU1 replication is dependant upon IHF (Hong-Yeoul Kim, personal communication). The region to the right of the second *SspI* site also contains over

half of the AT-rich region, a feature common to replication regions. The loss of these sequences in pBP11 and pSP14 may be seen as a barrier to complementary strand replication in the recipient. If this is the case, then one would conclude that in all instances, these two plasmids were conducted as the co-integrate proposed by union at *nic* sites (Figure 4.15a) and that those transconjugants showing unaltered separate *oriT* plasmids were the result of recombination between *oriT* sequences as outline in Figure 4.15b. Sequences involved in complementary strand synthesis in the donor and recipient cell could be investigated by determining their ability initiate the conversion of a single strand phage genome to the double stranded form and done with the RP4 *oriT* region by Yakobson *et al.* (1990).

From the preceding discussion a functional map dividing the *oriT* region into three regions or domains can be proposed (refer to Figure 5.1). The region containing *nic* lies in the 65 bp sequence between the *Bsp*MI and *Ssp*I₂ sites. The region to the right of *nic* would be involved in complementary strand synthesis in the donor and recipient cells while the region to the left appears to be required for efficient *in vivo* nicking and therefore may represent nicking recognition functions. The extent of this left hand region is in question with respect to the thirteen, 11 bp direct repeats extending from nucleotide 201-376.

The results discussed above suggest that the *oriT* region of pCU1 does encode a site which is nicked *in vivo*. Attempts to demonstrate a relaxation complex however were unsuccessful. Together these results suggest that the relaxation process of pCU1 may differ from that found for other conjugative and non-conjugative plasmids.

Several hypothesis can be put forward to explain the lack of a conventional

relaxation complex. First, pCU1 may possess a relaxation complex which is not activated by either SDS or pronase, or which in addition to being inactivated by pronase as seen with RK2 may also be inactivated by SDS. Second, the relaxation complex may not be constitutively expressed, but may require induction possibly via the formation of stable mating pairs. Thirdly, isolation of a pCU1 relaxation complex may not be possible. Relaxation and rejoining at *nic* may occur constantly such that at any given time, the plasmid exists in both supercoiled and relaxed form rather than a complexed and non-complexed supercoiled form. A second possible hindrance to the isolation of a relaxation complex may be the involvement of a membrane bound protein in the complex which would allow nicking to occur only in conjunction with the membrane bound DNA. This would prevent the protein-DNA complex from being isolated from a cleared lysate preparation.

The results of the mobilization experiments involving the pMUR plasmids indicate that there are conjugal DNA metabolism functions which are not interchangeable between all of the plasmids encoding N *tra* systems. Efficient mobilization of two pCU1 derivatives pBGL and pCU56 from the same cell, demonstrated that these functions are encoded by DNA outside the pili region, clockwise from the *Bgl*III site at 3.3 Kb on the pCU1 map to the end of *tra*. Within this region, there appear to be at least three separate conjugative functions, the region next to *oriT* (pKM101 *traK*), the pKM101 *traJ* and the region carried on the *Bgl*III fragment extending from 3.3-7 Kb on the pCU1a map (pKM101 *traH* and *traI*). Only the *traJ* function of pKM101 can be complemented in *trans* by the pMUR plasmids.

Mobilization of the pCU1 derivatives and the pKM101::Tn5 mutants by the

Figure 5.1. Summary diagram of the *oriT* region of pCU1. Heavy solid line at top represents the 669 bp pCU1 *oriT* region. Lines below represent relevant deletion derivative. Mobilization frequencies are given to the right of each derivative along with results indicating whether the derivative showed evidence of donation in the form of independent transfer and/or co-integrate formation via site-specific recombination at *nic*. Sequence features are shown at the bottom of the figure. Below this the *oriT* region is divided into the three proposed functional domains. Broken line indicates uncertainty in the involvement of the 13 direct repeats in conjugal transfer. NA, not applicable, ND, not done, ++, 13-75% loss of pCU101 in transconjugants, +, less than 10% loss of pCU101 in transconjugants.

Plasmid	Mobilization frequency by pCU1	Independent transfer	Site specific nicking
pSP25	10 ⁻¹	ND	ND
pSP12	10 ⁻²	+	-
pSP19	10 ⁻³	++	-
pSP20	10 ⁻⁶	+	-
pSP18	10 ⁻⁵	-	-
pSP11	10 ⁻⁷	-	-
pBP11	10 ⁻⁶	++	+
pSP23	10 ⁻⁷	-	-
pSP22	10 ⁻⁶	-	-
pSP13	10 ⁻⁵	-	-

inverted repeats
 direct repeat
 AT rich region
 putative IHF binding sites

recognition of
 nicking functions
 in vivo
 complementary strand
 synthesis

pMUR plasmids was similar. As seen with both the pKM101*traK* mutant and pCU1 derivatives pBP1 and pBGL, the loss of the *trans* acting function(s) encoded by the region directly next to the *oriT*, results in the most significant loss of mobility by the pMUR plasmids. In pCU1, addition of the region extending to the *Bgl*II site at 7 Kb increased the mobility of pCU56 by a factor of 3 over that of pBP1. These results correspond well to results for both *traH* and *traI* mutants of pKM101. It is apparent that the *tra* functions encoded by the region within the *Bgl*II fragment extending from 3.3-7 Kb on the pCU1 map or *traI* or *traH* on pKM101 are either less essential to conjugal DNA metabolism than the region adjacent to the *oriT*, or are complemented to some extent by the pMUR plasmids while pKM101 *traK* is not.

The inability of pCU1 derivatives carrying small deletions into either end of the *Bgl*II fragment extending from 3.3-7 Kb to complement each other for mobilization in the presence of the pMUR plasmids, suggests that unlike pKM101 there is only one complementation region within this fragment. The complementation results are supported by the similarity mobilization frequencies of derivatives carrying deletions into either end of this fragment.

Efficient mobilization of the *traJ* mutant suggests that either the *traJ* function is not necessary for conjugal DNA metabolism during transfer by the pMUR plasmids or that the pMUR plasmids produce a similar gene product. This latter possibility is reminiscent of the *traZ* (also called *traI*^{*}) product of the F *tra* systems. Within the F *tra* systems there are only two *traZ* alleles while there are four *oriT/traY/traM* alleles. Within the scheme proposed for conjugal DNA metabolism, *traZ* is believed to act as the nicking enzyme which works in conjunction with the *traY* product. The TraY protein is believed to recognize *bom* and it is the recognition function which is

believed to be plasmid specific. In the case of pCU1, the high specificity of the region directly next to the *oriT* region (pKM101 *traK*) is an ideal candidate for the recognition functions. Within this hypothesis, the inability of pKM101 *traJ* to be complemented by other transfer systems would suggest that it functions specifically with the other *mob* functions of the N *tra* system.

The inability to demonstrate relaxation at the *oriT* prevents the use of this system to investigate the role of the three *tra* functions showing plasmid specificity in the nicking process. However, it should be possible to develop an *in vivo* nicking system based on the alteration events seen with pBP11 and pSP14. The instability of pCU101 could be used in a type of 'rescue' experiment. Mob⁻ pCU101 derivatives could be introduced into a RecA⁻ host containing pBP11. After an extended incubation period without selection for the pCU101 antibiotic marker, cells still maintaining the pCU101 marker could be isolated and screened for altered forms similar to those identified in this study.

6. CONCLUSIONS

1. The minimal *oriT* region of the conjugative plasmid pCU1 is 669 bp in size; however, this region can be reduced to 323 bp with only a two to three fold reduction in mobilization frequency.
2. The predominant sequence structures within the pCU1 *oriT* region include:
 - i) one set of thirteen 11 bp direct repeats.
 - ii) one set of two 14 bp direct repeats.
 - iii) six different inverted repeat sequences ranging from 6-10 bp in size
 - iv) two sequences showing consensus to the IHF binding sequence
 - v) two AT-rich sequences, 19 and 81 bp in size.
3. pCU1 could not be shown to encode a relaxation complex typical of other transfer systems; however, an *in vivo* nicking event within the *oriT* region was demonstrated.
4. Within the group of plasmids encoding N type transfer systems, there is plasmid specificity for functions which are not involved in pili formation.

7. REFERENCES

- Abdel-Monem, M., G. Taucher-Scholz and M. Klinkert. 1983. Identification of *Escherichia coli* DNA helicase I as the *traI* product of the F sex factor. *Proc. Natl. Acad. Sci. USA.* 80:4659-4663.
- Ando, T. and T. Arai. 1981. Genetic structure of the IncN plasmid N3. *Plasmid* 6:293-301.
- Auerswald, E.A., G. Ludwig and H. Schaller. 1979. Structural analysis of Tn5. *Cold Spring Harbour Symp. Quant. Biol.* 45:107-113.
- Bastia, D. 1978. Determination of restriction sites and the nucleotide sequence surrounding the relaxation site of ColE1. *J. Mol. Biol.* 124:601-639.
- Berg, D.E., M.A. Schmandt and J.B. Lowe. 1983. Specificity of transposon Tn5 insertion. *Genetics* 105:813-828.
- Blair, D.G and D.R Helinski. 1975. Relaxation complexes of plasmid DNA and protein. I. Strand-specific association of protein and DNA in the relaxed complexes of plasmids ColE1 and ColE2. *J. Biol. Chem.* 250:8785-8789.
- Bolivar, F., R.L. Rodriguez P.J. Greene, M.C. Betlach, H.L. Haynecher, H.W. Boyer, J.H. Croso and S. Falkow. 1977. Construction and characterization of new cloning vehicles, II. A multipurpose cloning system. *Gene* 2:95-113.
- Bradley, D.E. 1979. Morphology of pili determined by the N incompatibility group plasmid N3 and interaction with bacteriophages PR4 and I_{ke}. *Plasmid* 2:632-636.
- Bradley, D.E. 1980. Morphological and serological relationships of conjugative pili. *Plasmid* 4:155-169.

- Bradley, D.E. 1981. Conjugative pili of plasmids in *Escherichia coli* K-12 and *Pseudomonas* species. In Molecular biology, pathogenicity and ecology of bacterial plasmids. (eds) S.B. Levy, R.C. Clowes and E.L. Koeing. Plenum Press. New York. pp. 217-226.
- Bradley, D.E., D.E. Taylor and D.R. Cohen. 1980. Specification of surface mating systems among conjugative drug resistance plasmids in *Escherichia coli* K-12. J. Bacteriol. 143:1466-1470.
- Brasch, M.A. and R.J. Meyer. 1987. A 38 base-pair segment of DNA is required in *cis* for conjugative mobilization of broad host range plasmid R1162. J. Mol. Biol. 198:361-369.
- Clark, A.J. and G.J. Warren. 1979. Conjugal transmission of plasmids. Ann. Rev. Genet. 13:99-125.
- Clewell, D.B. and D.R. Helinski. 1969. Supercoiled circular DNA-protein complex in *Escherichia coli*: purification and induced conversion to an open circular DNA form. Proc. Natl. Acad. Sci. USA. 62:1159-1166.
- Clewell, D.B. and D.R. Helinski. 1970. Properties of a supercoiled deoxyribonucleic acid-protein relaxation complex and strand specificity of the relaxation event. Biochem. 9:4428-4440.
- Covarrubias, L., L. Cervantes, A. Covarrubias, X. Soberon, I. Vichido, A. Blanco, Y.M. Kupersztoch-Portnoy and F. Bolivar. 1981. Construction and characterization of new cloning vehicles V. Mobilization and coding properties of pBR322 and several deletion derivatives including pBR327 and pBR328. Gene 13:25-35.
- Coupland, G.M., A.M.C. Brown and N.S. Willetts. 1987. The origin of transfer (*oriT*) of the conjugative plasmid R46: Characterization by deletion analysis and

- DNA sequencing. *Mol. Gen. Genet.* 208:219-225.
- Craig, N.L. and H.A. Nash. 1984. *E. coli* integration host factor binds to specific sites in DNA. *Cell* 39:707-716.
- Datta, N. 1974. Epidemiology and classification of plasmids. In *Microbiology- 1974*. D. Schlessinger (ed). Am. Soc. Microbiol. Washington. pp. 9-15
- Datta, N. and V.M. Hughes. 1983. Plasmids of the same Inc groups in *Enterobacteria* before and after the medical use of antibiotics. *Nature* 306:616-617.
- Davidson, N., R.C. Deonier, S. Hu and E. Ohtsubo. 1975. Electron microscope heteroduplex studies of sequence relations among plasmids of *Escherichia coli*. In *Microbiology-1974*. D. Schlessinger (ed). Am. Soc. Microbiol. Washington. pp. 56-65.
- Dente, L., G. Cesareni and R. Cortese. 1983. pEMBL: a new family of single stranded plasmids. *Nuc. Acids Res.* 11:1645-1655.
- Derbyshire, K.M. and N.S. Willetts. 1987. Mobilization of the non-conjugative plasmid RSF1010: A genetic analysis of its origin of transfer. *Mol. Gen. Genet.* 206:154-160.
- Dempsey, W.B. 1987. Integration host factor and conjugative transfer of the antibiotic resistance plasmid R100. *J. Bacteriol.* 169:4391-4392.
- Dempsey, W.B. and B.E. Fee. 1990. Integration host factor affects expression of two genes at the conjugal transfer origin of plasmid R100. *Mol. Microbiol.* 4:1019-1028.
- Dennison, S. and S. Baumberg. 1975. Conjugal behaviour of plasmids in *Escherichia coli* K12. *Mol. Gen. Genet.* 138: 323-331.

- Dobritsa, A.P., T.G. Mikhailova and V.I. Dubovaya. 1985. Physical and genetic structure of the IncN plasmid R15. *Plasmid* 14:99-105.
- Eckhardt, T. 1978. A rapid method for the identification of plasmid deoxyribonucleic acid in bacteria. *Plasmid* 1:534-588.
- Everett, R. and N. Willetts. 1980. Characterisation of an *in vivo* system for nicking at the origin of conjugal DNA transfer of the sex factor F. *J. Mol. Biol.* 136:129-150.
- Finlay, B.B., L.S. Frost and W. Paranchych. 1986. Nucleotide sequences of the R1-19 plasmid transfer genes *traM*, *finP*, *traJ*, *traY*, and the *traYZ* promoter. *J. Bacteriol.* 166:368-374.
- Finlay, B.B., L.S. Frost and W. Paranchych. 1986. Origin of transfer of IncF plasmids and nucleotide sequences of the type II *oriT*, *traM*, and *traY* alleles from ColB4-K93 and the type IV *traY* allele from R100-1. *J. Bacteriol.* 168:132-139.
- Freidman, D.I., E.J. Olson, D. Carver and M. Gellert. 1984. Synergistic effect of *himA* and *gyrB* mutations: evidence the Him functions control express of *ilv* and *xyl* genes. *J. Bacteriol.* 157:484-489.
- Gamas, P., A.C. Burger, G. Churchward, L. Caro, D. Galas and M. Chandler. 1986. Replication of pSC101: effects of mutations in the *E. coli* DNA binding protein IHF. *Mol. Gen. Genet.* 204:85-89.
- Gray, H.B. Jr., D.A. Ostrander, J.L. Hodnett, R.J. Legerski and D.L. Robberson. 1975. Extracellular nucleases of *Pseudomonas* Bal31. I. Characterization of single strand-specific deoxyriboendonuclease and double-strand deoxyriboexonuclease activities. *Nuc. Acid Res.* 2:1459-1492.

- Goosen, N. and P. van de Putte. 1984. Regulation of Mu transposition. I. Localization of the presumed recognition sites for HimD and Ner functions controlling bacteriophage Mu transcription. *Gene* 30:41-46.
- Guerry, P., J. Van Embden and S. Falkow. 1974. Molecular nature of two non-conjugative plasmids carrying drug resistance genes. *J. Bact.* 117:619-630.
- Guiney, D.G., C. Deiss and V. Simnad. 1988. Location of the relaxation complex nick site with the minimal origin of transfer region of RK2. *Plasmid* 2:259-265.
- Guiney, D.G. and D.R. Helinski. 1975. Relaxation complexes of plasmid DNA and protein. III. Association of protein with the 5' terminus of the broken DNA strand in the relaxed complex of plasmid ColE1. *J. Biol. Chem.* 250:8796-8803.
- Guiney, D.G. and D.R. Helinski. 1979. The DNA-Protein relaxation complex of the plasmid RK2: Location of the site specific nick in the region of the proposed origin of transfer. *Mol. Gen. Genet.* 176:183-189.
- Guiney, D.G. and E. Jakobson. 1983. Location and nucleotide sequence of the transfer origin of the broad host range plasmid RK2. *Proc. Natl. Acad. Sci. USA.* 80:3595-3598.
- Holmes, D.S. and M. Quigley. 1981. A rapid boiling method for the preparation of bacterial plasmids. *Anal. Biochem.* 114:193-197.
- Horowitz, B. and R.C. Deonier. 1985. Formation of Δtra F' plasmids: specific recombination at *oriT*. *J. Mol. Biol.* 186:267-274.
- Hoyt, M.A., D.M. Knight, A. Das, H.I. Miller, H. Echols. 1982. Control of phage λ development by stability and synthesis of *cII* protein: role of the viral *chi* and

- host *hfiA*, *himA*, and *himD* genes. *Cell* 31:565-573.
- Hughes, V.M. and N. Datta. 1983. Conjugative plasmids in bacteria of the 'pre-antibiotic' era. *Nature* 302: 725-726.
- Inselburg, J. 1977. Studies of colicin E1 plasmid function by analysis of deletions and *TnA* insertions of the plasmid. *J. Bacteriol.* 132:332-340.
- Ippen-Ihler, K.A. and E.G. Minkley, Jr. 1986. The conjugation system of F, the fertility factor in *Escherichia coli*. *Ann. Rev. Genet.* 20:593-624.
- Ippen, K.A. and R.C. Valentine. 1967. The sex hair of *E. coli* as a sensory fibre, conjugation tube or mating arm. *Biochem. Biophys. Res. Comm.* 27:674-680.
- Iyer, V.N. 1989. IncN group plasmids and their genetic systems. *In* Promiscuous plasmids of gram-negative bacteria. Academic Press. London. pp. 165-183.
- Jacobson, A. 1972. Role of F pili in the penetration of bacteriophage f1. *J. Virol.* 10:835-843.
- Johnson, D., R. Everett and N. Willetts. 1981. Cloning of F DNA fragments carrying the origin of transfer *oriT* and the fertility inhibition gene *finP*. *J. Mol. Biol.* 153:187-202.
- Kahn, M., R. Kolter, C. Thomas, D. Figurski, R. Meyer, E. Remaut and D.R. Heninski 1979. Plasmid cloning vehicles derived from plasmids ColE1, F, R6K and RK2. *Methods Enzymol.* 68:268-280.
- Khatoon, H., R.V. Iyer and V.N. Iyer. 1972. A new filamentous bacteriophage with sex-factor specificity. *Virol.* 48:145-155.
- Kilbane, J.J. and M.H. Malamy. 1980. F factor mobilization of non-conjugative chimeric plasmids in *Escherichia coli*: general mechanisms and a role for site-specific *recA*-independent recombination at *oriV1*. *J. Mol. Biol.* 143:73-93.

- Kline, B.C. and D.R. Helinski. 1971. F_1 sex factor of *Escherichia coli*. Size and purification in the form of a strand-specific relaxation complex of supercoiled deoxyribonucleic acid and protein. *Biochem.* 10:4975-4980.
- Komano, T., A. Toyoshimam, K. Morita and T. Nisioka. 1988. Cloning and nucleotide sequence of the *oriT* region of the IncI1 plasmid R64. *J. Bact.* 170:4385-4387.
- Konarska-Kozłowska, M. and V.N. Iyer. 1983. Sequence homology between IncN group plasmids. *Plasmid* 10:211-223.
- Krishnan, B.R. and V.N. Iyer. 1990. IncN plasmid replicon: a deletion and subcloning analysis. *J. Mol. Biol.* 213:777-788.
- Kues, U. and U. Stahl. 1989. Replication of plasmids in gram-negative bacteria. *Microbiol. Rev.* 53:491-515.
- Kupersztoch-Portnoy, Y.M., M.A. Lovett and D.R. Helinski. 1974₁. Strand and site specificity of the relaxation event for the relaxation complex of the antibiotic resistance plasmid R6K. *Biochem.* 13:5484-5490.
- Kupersztoch-Portnoy, Y.M., G.L.G. Miklos and D.R. Helinski. 1974₂. Properties of the relaxation complexes of supercoiled deoxyribonucleic acid and protein of the R plasmids R64, R28K and R6K. *J. Bacteriol.* 120:545-548.
- Lahue, E.E. and S.W. Matson. 1990. Purified *Escherichia coli* F-Factor TraY protein binds at *oriT*. *J. Bacteriol.* 172:1385-1391.
- Lanka, E. and P.T. Barth. 1981. Plasmid RP4 specifies a deoxyribonucleic acid primase involved in its conjugal transfer and maintenance. *J Bact.* 148:769-781.
- Lawn, A.M., E. Meynell, G.G. Meynell, N. Datta. 1967. Sex pili and the classification

- of sex factors in the Enterobacteriaceae. *Nature* 216:343-346.
- Leong, J.M., S. Nunes-Duby, C.F. Lesser, P. Youderian, M.M. Susskind and A. Landy
1985. The $\phi 80$ and P22 attachment sites. Primary structure and interaction
with *Escherichia coli* integration host factor. *J. Biol. Chem.* 260:4468-4477.
- Liss, L.R. 1987. New M13 host: DH5 α F' competent cell. *Focus* 9:13.
- Lovett, M.A., D.G. Guiney and D.R. Helinski. 1974. Relaxation complexes of
plasmids ColE1 and ColE2: unique site of the nick in the open circular DNA
of the relaxed complexes. *Proc. Natl. Acad. Sci. USA.* 71:3854-3857
- Lovett, M.A and D.R. Helinski. 1975. Relaxation complexes of plasmid DNA and
protein. II. Characterization of the proteins associated with the unrelaxed and
relaxed complexes of plasmid ColE1. *J. Biol. Chem.* 250:8790-8795.
- McIntire, S.A. and W.B. Dempsey. 1987. *oriT* sequence of the antibiotic resistance
plasmid R100. *J. Bacteriol.* 169:3829-3832.
- Messing, J. 1983. New M13 vectors for cloning. *Methods Enzymol.* 101:20-79.
- Nash, H.A. and C.A. Robertson. 1981. Purification and properties of the
Escherichia coli protein factor required for lambda integrative recombination
J. Biol. Chem. 256:9246-9253.
- Nijkamp, H.J.J., R. DeLang, A.R. Stuitj, P.J.M. Van Den Elzen, E. Veltkamp and A.J
Van Putten. The complete nucleotide sequence of the bacteriocinogenic
plasmid CloDF13. *Plasmid* 16:135-160
- Nordheim, A., T. Hashimoto-Gotoh and K.N. Timmis. 1980. Location of two
relaxation nick sites in R6K and single sites in pSC101 and RSF1010 close to
origins of vegetative replication: implication for conjugal transfer of plasmid
deoxyribonucleic acid. *J. Bact.* 144:923-932.

- O'Connor, M.B. and M.H. Malamy. 1984. Site-specific recombination in the *oriV* region of the F factor. Cold Spring Harbor Symp. Quant. Biol. 49:421-434
- Olsen, R.H. and P.L. Shipley. 1975. RP1 properties and fertility inhibition among P, N, W, and X incompatibility group plasmids. J. Bacteriol. 123:28-35.
- Ostermann, E., F. Kricek and G. Hogenauer. 1984. Cloning the origin of transfer region of the resistance plasmid R1. EMBO J. 3:1731-1735.
- Panicker, M.M. and E.G. Minkley, Jr. 1985. DNA transfer occurs during a cell surface contact stage of F sex factor-mediated bacterial conjugation. J. Bacteriol. 162:584-590.
- Phadnis, S.H. and D.E. Berg. 1987. Identification of base pairs in the outside end of insertion sequence IS50 that are needed for IS50 and Tn5 transposition. Proc. Natl. Acad. Sci. USA. 84:9118-9122.
- Pinney, R.J. and J.T. Smith. 1974. Fertility inhibition of an N group R factor by a group X R factor, R6K. J. Gen. Microbiol. 82:415-418.
- Remington, R.D. and M.A. Stork. 1970. Statistics with applications to the biological and health sciences. Prentice Hall Inc. Englewood Cliff.
- Rothstein, S.J., R.A. Jorgensen, J.C.P. Yin, Z. Yong-Di, R.C. Johnson and W.S. Reznikoff. 1981. Genetic organization of Tn5. Cold Spring Harbour Symp. Quant. Biol. 45:99-105.
- Schaffer, H.E. and R.R. Sederoff. 1981. Improved estimation of DNA fragment lengths from agarose gels. Anal. Biochem. 115:113-122.
- Sims, J. and E.W. Benz Jr. 1980. Initiation of DNA replication by the *Escherichia coli* *dnaG* protein: evidence that tertiary structure is involved. Proc. Natl. Acad. Sci. USA 77:900-904.

- Snijder, A., A.J. van Putten, E. Veltkamp and H.J.J. Nijkamp. 1983. Localization and nucleotide sequence of the *bom* region of CloDF13. *Mol. Gen. Genet* 192:444-451.
- Tanimoto, K and T. Iino. 1983. Transfer inhibition of RP4 by F factor. *Mol. Gen. Genet.* 192:104-109.
- Thatte, V., D.E. Bradley and V.N. Iyer. 1985₁. N conjugative transfer system of plasmid pCU1. *J. Bacteriol.* 163:1229-1236.
- Thatte, V., S. Gill and V.N. Iyer. 1985₂. Regions on plasmid pCU1 required for the killing of *Klebsiella pneumoniae*. *J. Bacteriol.* 163:1296-1299
- Thatte, V. and V.N. Iyer. 1983. Cloning of a plasmid region specifying the N transfer system of bacterial conjugation in *Escherichia coli*. *Gene* 21:227-236.
- Thompson, R. and M. Achtman. 1979. The control region of the F sex factor DNA transfer cistrons: physical mapping by deletion analysis. *Mol. Gen. Genet* 169:49-57.
- Thompson, R., L. Taylor, K. Kelly, R. Everett and N. Willetts. 1984. The F plasmid origin of transfer: DNA sequence of wild-type and mutant origins and location of origin specific nicks. *EMBO* 3:1175-1180.
- Thompson, T.L., M.B. Centola and R.C. Deonier. 1989. Location of the nick at *oriT* of the F plasmid. *J. Mol. Biol.* 207:505-512.
- Traxler, B.A. and E.G. Minkley, Jr. 1987. Revised genetic map of the distal end of the F transfer operon: implications for DNA Helicase I, nicking at *oriT*, and conjugal DNA transport. *J. Bacteriol.* 169:3251-3259.
- Tasi, M-M., Y-H. F. Fu and R.C. Deonier. 1990. Intrinsic bends and integration host

- factor binding at F plasmid *oriT*. J. Bacteriol. 172:4603-4609.
- van de Pol, H., E. Veltkamp and H.J.J. Nijkamp. 1978. Genetic analysis of the mobilization of the non-conjugative plasmid CloDF13. Mol. Gen. Genet. 160:139-149.
- Vieira, J. and J. Messing. 1987. Production of single-stranded plasmid DNA. Methods Enzymol. 153:3-11.
- Warren, G.J. and A.J. Clark. 1979. Conjugal transmission of plasmids. Ann. Rev. Genet. 13:99-125.
- Warren, G.J. and A.J. Clark. 1980. Sequence-specific recombination of plasmid ColE1. Proc. Natl. Acad. Sci. USA. 77:6724-6728.
- Warren, G.J., M.W. Saul and D. J. Sherratt. 1979. ColE1 plasmid mobility: essential and conditional functions. Mol. Gen. Genet. 170:103-107.
- Warren, G.J., A.J. Twigg and D.J. Sherratt. 1978. ColE1 plasmid mobility and relaxation complex. Nat. 274:259-261.
- Willetts, N. 1980. Interactions between the F conjugal transfer system and CloDF13::TrnA plasmids. Mol. Gen. Genet. 180:213-217.
- Willetts, N. 1981. Sites and systems for conjugal DNA transfer in bacteria. in Molecular biology, pathogenicity and ecology of bacterial plasmids. S.B. Levy, R.C. Clowes and E.L. Koeing (eds). Plenum Press. New York. pp. 207-215.
- Willetts, N. and C. Crowther. 1981. Mobilization of the non-conjugative IncQ plasmid RSF1010. Genet. Res. 37:311-316.
- Willetts, N. and J. Maule. 1985. Specificities of IncF plasmid conjugation genes. Genet. Res. 47:1-11.
- Willetts, N. and R. Skurray. 1980. The conjugation system of F-like plasmids. Ann.

- Rev. Genet. 14:41-76.
- Willetts, N. and B. Wilkins. 1984. Processing of plasmid DNA during bacterial conjugation. Microbiol. Rev. 48:24-41.
- Winans, S.C. and G.C. Walker. 1985₁. Fertility inhibition of RP1 by IncN plasmid pKM101. J. Bacteriol. 161:425-427.
- Winans, S.C. and G.C. Walker. 1985. Conjugal transfer system of the IncN plasmid pKM101. J. Bacteriol. 161:402-410.
- Yakoboson, E., C. Deiss, K. Hirata and D.G. Guiney. 1990. Initiation of DNA synthesis in the transfer origin region of RK2 by the plasmid-encoded primase: detection using defective M13. Plasmid 23:80-84.
- Yakoboson, E. and G. Guiney. 1983. Homology in the transfer origins of broad host range IncP plasmids: definition of two subgroups of P plasmids. Mol. Gen. Genet. 192:436-438.
- Ziegelin, G., J.P. Furste and E. Lanka. 1989. TraJ protein of plasmid RP4 binds to a 19-base pair invert sequence repetition with the transfer origin. J. Biol. Chem. 264:11989-11994.

X-gal 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside

YT Yeast tryptone broth

μ Ci microcurri(es)

μ g microgram(s)

μ l microlitre(s)

μ M micromolar

END

04.03.92

FIN