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**THE IncN ANTIBIOTIC-RESISTANCE PLASMID pCU1:
BACTERIAL HOST-RANGE, NUCLEOTIDE SEQUENCE, DELETION
AND SUBCLONING ANALYSIS OF THE REPLICON**

by

B. Rajendra Krishnan

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

Department of Biology

Carleton University

Ottawa, Ontario

1989

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the Faculty of Graduate Studies and Research
acceptance of the thesis,
**"The IncN Antibiotic-Resistance Plasmid pCU1:
Bacterial Host-Range, Nucleotide Sequence, Deletion and
Subcloning Analysis of the Replicon"**
submitted by
B. Rajendra Krishnan
in partial fulfilment of the requirements
for the degree of Doctor of Philosophy

Chairman, Department of Biology

Thesis Supervisor

External Examiner

Carleton University

Abstract

The bacterial host ranges of the conjugatively self-transmissible IncN group plasmid pCU1 and its miniderivative (constructed to contain the plasmid's replicon and the *oriT* DNA sequences of the broad-host-range plasmid RK2) were examined. Species of Gram-negative purple bacteria were chosen for this study. The host-range of the IncN *tra* system is broader than that of its replicon. Within the γ -subdivision of purple bacteria, the host ranges of the two plasmids were similar.

A procedure for the detection of the autonomous-replication ability of a cryptic DNA fragment is described. This was used to demonstrate unambiguously that a 2 kb *Pvu*II DNA fragment of pCU1 (previously concluded to contain its replicon, Kozlowski *et al.*, 1987) contained sufficient genetic determinants to exhibit autonomous and stable existence.

An examination of the primary structure of the 2 kb *Pvu*II fragment of pCU1 revealed the following features (a) the presence of two sets of direct iterons (a family of thirteen 37 bp tandem and a second family of two 30 bp non-tandem repeats) (previously noted by Kozlowski *et al.*, 1987), (b) the presence of fourteen putative *dnaA* boxes and four 'GATC' sequences and (c) the presence of six open reading frames (ORFs) encoding putative polypeptides of 46, 68, 188, 203, 239 and 245 amino acids respectively. A role for the tandem iterons and ORFs 68 and 245 in plasmid replication was excluded by deletion and subcloning analysis. A DNA segment contains the basic-replicon of pCU1 within a 1019 bp region. Polypeptide analysis suggested that ORF239 and ORF46 participate in plasmid replication. Two independent polypeptide chain-terminating mutations in ORF239 abolished the *E. coli polA*-independent mode of replication. However, under these circumstances, the plasmid adopts a *polA*-dependent mode of replication.

The *cis*-acting region(s) of the pCU1 replicon was localized to 368 bp that includes an imperfect *dnaA* box and three 'GATC' sequences. Maintenance of the pCU1 replicon

was shown to be *E. coli dnaA*-dependent. *E. coli* Dam-methylation of the pCU1 replicon enhanced stability.

Two DNA domains that express IncN incompatibility were identified within the replicon of pCU1. One of these includes members of the direct tandem iterons. A role for these iterons in the control of plasmid copy-number was also demonstrated.

"The goal of mankind is knowledge"

-Swami Vivekananda in 'Karma Yoga'

To my parents, with love

and

To my teachers, with gratitude

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List of Abbreviations

>	greater than
<	lesser than
ATP	Adenosine triphosphate
Ap	Ampicillin
bp	base pair(s)
°C	temperature in degrees Celsius
Cm	Chloramphenicol
cfu	colony forming unit(s)
<i>cop</i>	copy number control gene(s)
ccc	covalently closed circular
Ci	Curie(s)
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
ddNTP	Dideoxynucleotide triphosphate(s)
DNA	Deoxyribonucleic acid
ds	double-stranded
EDTA	Ethylenediamine tetra-acetic acid
EtBr	Ethidium bromide
Fig.	figure
gm	gram(s)
g	acceleration due to gravity
h	hour(s)
Inc	incompatibility group
IS	Inzertion sequence
IPTG	Isopropyl- β -D-thiogalactopyranoside
Km	Kanamycin
kb	kilobase pair(s)
LA	Luria agar

LB	Luria broth
mRNA	messenger RNA
mg	milligram(s)
ml	milliliter(s)
mM	millimolar
min	minute(s)
mob	mob'ilization (gene)
M	molar
MW	molecular weight in daltons
moi	multiplicity of infection
Nal	Nalidixic acid
NB	Nutrient broth
ng	nanogram(s)
nm	nanometer(s)
ORF	open reading frame
O.D.	optical density
<i>oriT</i>	origin of conjugative transfer
<i>oriV</i>	origin of vegetative replication
PA	Penassay agar
pfu	plaque forming unit(s)
<i>polA</i>	<i>E. coli</i> DNA polymerase I gene
PEG	Polyethylene glycol
+	presence of a phenotype
<i>rep</i>	replication gene(s)
RF	replicative form
r	resistance
RNA	Ribonucleic acid
rRNA	ribosomal RNA
Rif	Rifampicin
sec	second(s)
s	sensitivity

ss	single-stranded
SDS	Sodium dodecyl sulfate
sp.	species
Sp	Spectinomycin
Sm	Streptomycin
Ts	temperature-sensitive
Tc	Tetracycline
tRNA	transfer RNA
Tn	transposon
TBE	Tris-HCl, boric acid, EDTA buffer
TE	Tris-HCl, EDTA buffer
TAE	Tris-HCl, sodium acetate, EDTA buffer
TES	Tris-HCl, sodium chloride, EDTA buffer
<i>tra</i>	conjugal transfer gene(s)
U	unit(s)
ug	microgram(s)
ul	microliter(s)
UV	Ultra-violet light
V	Volts
v/v	volume per unit volume
w/v	weight per unit volume
X-gal	5-bromo-4-chloro-3-indolyl- β -D-galactoside

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CHAPTER 1

Introduction

and

A Review of the Literature

Bacterial plasmids are autonomously replicating extrachromosomal elements. They comprise a large group of diverse elements. A scheme of classification of plasmids of Gram-negative bacteria is based upon their expression of incompatibility (Jacob *et al.*, 1977). Incompatibility (Inc) is defined as the inability of two plasmids to be stably maintained together in a bacterial cell line in the absence of selective pressure (Nordstrom *et al.*, 1984). The basic replicons of plasmids of the same Inc group are generally closely related and exhibit similarities in overall genetic organization (see Thomas and Smith, 1987; Iyer, 1989). The IncN group of plasmids constitute one such group and have some interesting properties (see below). Plasmid pCU1 is a representative of this group (Konarska-Kozłowska and Iyer, 1981a). The subject of this thesis is pCU1 (Fig. 1.1) and specifically, its basic replicon - a relatively small region of the plasmid sufficient to maintain the plasmid state. This section begins with a brief overview of the IncN group plasmids and their genetic functions leading up to what is known about the IncN replicon. This is followed by a review of published information of other plasmid replicons so that the contents of the rest of the thesis can be placed in perspective. A wealth of information has been accumulated on bacterial plasmid replication. Here, only those replicons of plasmids that have been found naturally in Gram-negative bacteria and even among this group only those plasmid replicons on which there is detailed structural information (DNA sequence) available are compared.

1.1. BIOLOGY OF IncN GROUP PLASMIDS

IncN plasmids are generally characterized by their ability to confer sensitivity to bacteriophage IKE in cells carrying them (Khatoon *et al.*, 1972). They are conjugative plasmids and in nature usually (but not always) specify resistance to a number of antibiotics

Fig. 1.1. Genetic map of pCU1 (Konarska-Kozłowska and Iyer, 1981b; Thatte *et al.*, 1985a; Iyer, 1989)

pCU1 is a 39 kb IncN group plasmid. Ap, Sm and Sp indicate plasmid regions that specify ampicillin, streptomycin and spectinomycin resistances respectively. *tra* is the plasmid region that encodes functions for bacterial conjugation. *oriT* is the *cis*-acting part of the *tra* region and contains the site wherefrom conjugative transfer is initiated. *kikA* and *kikB* encode functions for the killing of *Klebsiella pneumoniae* during bacterial mating. *kim* contains plasmid functions specifying immunity to this mode of killing. The closed rectangle depicts the location of the IS46 element on the plasmid. *rep* contains all plasmid determined functions for the replication and stable maintenance of the pCU1 replicon as defined in *E. coli*. *rep* is the subject of study in this dissertation.

(for a detailed review see Iyer, 1989). The various genetic features of IncN plasmids are summarized below.

1.1.1. Host-range of IncN plasmids

IncN plasmids have been isolated in nature from *Escherichia*, *Klebsiella*, *Salmonella*, *Shigella*, *Proteus* and *Providencia* sp. (Shapiro, 1977) and have been transferred in the laboratory to *Caulobacter* (Ely, 1979) and *Myxococcus* (Parish, 1975) sp. Thus, they seem to exhibit a broad host-range among Gram-negative bacteria. Tardif and Grant (1980) showed that IncN plasmids were transferred to bacteria of the class Enterobacteriaceae at a high frequency and stably maintained in them. They were transferred at a low frequency to and maintained poorly in non-enteric bacteria. The first objective of this project was to determine the limits of host-range of a representative IncN group plasmid and of a mini-plasmid containing its replicon (Chapter 3). This would then indicate the plasmid region(s) that determine or contribute to its host-range.

1.1.2. Conjugative transfer region

Naturally isolated IncN plasmids are self-transmissible and specify a distinctive system of bacterial conjugation (Thatte and Iyer, 1983). The plasmid regions encoding such functions have been well-characterized in pCU1 and pKM101 (Thatte *et al.*, 1985a; Winans and Walker, 1985a). A contiguous region of plasmid DNA of approximately 15 kb contains all genetic information for plasmid transfer in pCU1 and conferred self-transmissibility to a heterologous, non-conjugative plasmid pACYC184 (Thatte and Iyer, 1983; Thatte *et al.*, 1985a). Transposon insertion and genetic complementation analysis of the *tra* region of pCU1 and pKM101 helped in the identification of eight transcriptional units in pCU1 and

eleven in pKM101. Further, remarkable similarities were observed in the genetic organization and in the distribution of restriction sites within the *tra* regions of pCU1 and pKM101 (Iyer, 1989). The *tra* region contains a *cis*-acting site for plasmid transfer, *oriT*. The DNA sequence of *oriT* of plasmid R46 (pKM101 is a deletion derivative of R46, Langer and Walker, 1981) exhibits thirteen 11 bp direct tandem iterons (deoxyribonucleotide sequences that are repeated) and three different pairs of indirect 10 bp iterons (Coupland *et al.*, 1987). These structural features are unique to the *oriT* of the IncN group and are not shared by the *oriT* regions of plasmids of other Inc groups whose DNA sequences have been established so far (Iyer, 1989).

1.1.3. Plasmid regions that encode host-lethality

Two genes, *kilA* and *kilB* have been mapped on the pKM101 genome (Winans and Walker, 1985b). Expression of these genes results in the killing of the host *E. coli* and is overridden by the expression of the *korA* and *korB* genes. Expression of both *korA* and *korB* is required to override expression of either *kilA* or *kilB*. *kilB* is part of the *traD* operon of the conjugative transfer system.

IncN plasmids also mediate killing of recipient *Klebsiella pneumoniae* cells in a bacterial conjugation system (Rodriguez and Iyer, 1981). Two regions, *kikA* and *kikB* (*kik*: killing of *Klebsiella*) were mapped on the pCU1 genome (Thatte *et al.*, 1985b) whose coordinate expression is required for *Klebsiella* killing. Insertion mutations within *kikA* abolished the *Kik*⁺ phenotype and also rendered the host cell PRD1^r (PRD1 is a bacteriophage; IncN plasmids confer sensitivity to both Ike and PRD1). This suggests that the genetic region *kikA* probably has a dual role. None of the Tn5 insertions mapped

within *kikB* altered the Kik^+ phenotype indicating that *kikB* probably does not encode a product that participates in the Kik^+ phenotype. Instead it could play a regulatory role while *kikA* is the structural gene required for killing. Nevertheless, simultaneous transfer of both the *kikA* and *kikB* regions *in cis* or *in trans* is required for the expression of the killing function (Thatte *et al.*, 1985b). Recently, it was shown that none of the gene products of the *tra* system contributed to the killing of *Klebsiella* (Rotheim *et al.*, 1988). This does not however, exclude the possibility that bacterial mating is required for the expression of the Kik^+ phenotype.

In a killing reaction 1-10% of the recipient *Klebsiella* cells survive. A *Klebsiella* cell that has once 'experienced' pCU1 is 'immune' to any further killing by pCU1. This immunity is imprinted in the *Klebsiella* genome even after plasmid curing. The nature of the 'mutation' in such immune *Klebsiella* is not established. Integrated pCU1 DNA could not be detected in the immune *Klebsiella* cells (Gill, 1985). Further, a pCU1 specified region, *kim*, has been mapped, the establishment of which in recipient *Klebsiella* using heterologous plasmid vectors confers immunity to *Klebsiella*. The mechanisms of these immunity functions remain a mystery (Gill, 1985).

1.1.4. Plasmid regions encoding essential replication functions

As in the other plasmids (Section 1.2), the plasmid region(s) required for autonomous replication of IncN plasmids is confined to a small segment of the plasmid genome. Deletion mapping showed that an approximately 2.5 kb region of the 35.4 kb pKM101 (and therefore of the 51.7 kb R46) contains all plasmid determined *rep* functions (Langer *et al.*, 1981). In R15, the *rep* region is contiguous with the plasmid's *tra* region and is contained within nucleotide co-ordinates 46.0-53.7 in the 62.3 kb map of the plasmid

(Dobrista *et al.*, 1985) Two regions expressing autonomous replication ability were identified in the 54 kb plasmid N3 (Ando and Arai, 1981). The *replI* and *replII* regions were cloned into pBR322 or ColE1 plasmids. While the recombinant derivative containing *replI* was stably maintained when transferred to an *E.coli polA* strain p3478, the derivative containing *replII* was rapidly lost after transfer. The minimal replication region of pCU1 is contained in a unique 2 kb *PvuII* DNA fragment of the plasmid and the complete nucleotide sequence of this segment is known (Kozlowski *et al.*, 1987). A summary of the characteristic features of this replicon is depicted in Fig. 1.2. Two families of direct iterons of thirteen (Group I) and two (Group II repeats) members were recognized. Group I repeats contain 37 bp each and those of Group II contain 30 bp each. A 19 bp DNA sequence contained partially within the left member of the Group II repeats and partially outside it is represented in an inverted fashion straddling each member of the Group I repeats. Potentially, this could form intrastrand hairpin loops. Further, three major open reading frames (ORFs) were initially deduced from the DNA sequence: ORF48, ORF57 and ORF207 that could encode putative polypeptides of 47, 56 and 206 amino acids respectively. ORF207 includes all members of the Group I repeats and hence, the putative polypeptide would be an unusual protein with a monotonous repeat of amino acids. Also, ORF207 exhibits inappropriately placed *E. coli* -10 consensus regulatory signals and a total lack of -35 promoter sequences (Kozlowski *et al.*, 1987). A comparison of the features of the IncN replicon exemplified by pCU1 to other well-characterized replicons of both broad host-range (RK2, pSa, RSF1010) and narrow host-range (F, P1, pSC101) (Section 1.2) abilities points out the uniqueness of the structural organization of the IncN replicon. A probe containing the replicon of pCU1 hybridized to the other IncN plasmids, R199, pDT200, pIE305, pIE330, pIE337, pIE512 and pIE514 (Konarska-Kozlowska and Iyer, 1983) suggesting that the basic features of the IncN replicon

Fig. 1.2. Summary of the salient features of the replicon of pCU1 (Kozlowski *et al.*, 1987)

The closed arrowheads indicate the thirteen 37 bp tandem direct iterons (Group I). The open arrowheads show the two 30 bp direct Group II iterons. A partial repeat of this family is also shown at the beginning of the second member of these iterons. Note the spacer region inbetween the Group II iterons. Discontinuous lines indicate regions that can form hairpin loop-structures (see text for details). Arrows depict the three open reading frames (ORFs) deduced from the DNA sequence. Restriction enzyme sites for *Pvu*II are also shown in the figure.

are conserved among the different IncN plasmids. Therefore, a second and major objective of this thesis was to construct a more precise structural map of an IncN replicon (Chapter 4) and attempt to ascribe functions to different parts of the replicon (Chapter 5). pCU1 was used as the representative IncN plasmid.

1.1.5. Other significant functions encoded by IncN plasmids

IncN group of plasmids are known to specify DNA restriction and modification activities (Arai and Aoki, 1977). One of such widely used systems is *EcoRII*. Winans and Walker (1983) showed that pKM101 carried a *nuc* gene contained within its *tra* region. The *nuc* gene encodes a DNA specific nuclease function but its precise role *in vivo* is unknown. Winans and Walker (1985 c, d) also mapped genes on pKM101 that (a) affect the conjugative functions of a co-resident IncP plasmid, (b) make the pKM101-carrying host a poor recipient for IncN plasmids in general and (c) contribute to the stability of the plasmid in *E. coli*. These genes were designated *fip* (fertility inhibition of *P* group plasmids), *eex* (entry exclusion) and *stb* (stability) respectively.

Insertion element IS46 has been mapped in the genomes of pCU1, R46 and N3 (Brown *et al.*, 1984). This may serve as a structure for recombination events. Indeed, a Tn4 like site-specific recombination system has been described for R46 (Dodd and Bennett, 1983).

The ability of IncN plasmids to enhance UV mutability of *E. coli* and *S. typhimurium* has been exploited in the development of the Ames test. The plasmid genes *mucA* and *mucB* that are functionally analogous to the bacterial genes *umuC* and *umuD* have been mapped on the pKM101 genome and well-characterized. The *mucAB* operon is under the

transcriptional control of the LexA repressor similar to its *umuCD* counterpart. Upon UV-induction, RecA cleaves LexA and *mucA* and *mucB* are expressed (reviewed by Iyer, 1989).

1.2. STRUCTURAL ORGANIZATION OF PLASMID REPLICONS

Plasmids in nature vary considerably in size from a few kb (e.g. ColE1) to greater than several hundreds of kb (e.g. Sym plasmid of *Rhizobium* sp.). Despite such large variations, the genetic region(s) for plasmid DNA replication is contained in a small fraction of the plasmid genome. All plasmids utilize some of the host-specified genetic function(s) for replication. However, some of them substitute several such factors with their own plasmid-encoded products. Examples of the *E. coli* chromosome-determined functions used by some of the plasmids are shown in Table 1.1.

Plasmid-determined function(s) can be broadly divided as the *cis*-acting region (which always includes an *origin*(s) region from which plasmid DNA replication is initiated) and a *trans*-acting region(s) that regulates initiation of replication. Plasmids may contain one origin of replication (as in the case of ColE1, Lovett *et al.*, 1974) or more than one origin (e.g. one of the replicons of F which contains two origins, Kline, 1985 and a derivative of R6K, RSF1040 which contains three origins, Crosa, 1980). There is only one plasmid encoded essential replication protein in replicons such as R6K (Germino and Bastia, 1982; Filutowicz *et al.*, 1985a) while there are three replication proteins encoded by the RSF1010 genome (Scherzinger *et al.*, 1984). No plasmid encoded proteins are required for the replication of ColE1 (Clewell, 1972). A detailed description of the organization of the origin(s) and the structural gene(s) in the various replicons follows and Figs. 1.3-1.7 and Table 1.2 provide a comparative summary.

Genetic markers of *E. coli*

Gene symbol	Gene product and/or Phenotypic trait
<i>dam</i>	DNA adenine methylase
<i>dnaA</i>	DNA replication initiation
<i>dnaB</i>	DNA helicase and replication initiation
<i>dnaC</i>	Initiation of DNA biosynthesis and chain elongation
<i>dnaE</i>	subunit of DNA polymerase III
<i>dnaG</i>	DNA primase
<i>dnaZ</i>	subunit of DNA polymerase III
<i>gyr</i>	DNA gyrase
<i>himA</i>	Integration host factor α subunit
<i>himD</i>	Integration host factor β subunit
<i>polA</i>	DNA polymerase I
<i>rpo</i>	RNA polymerase

Table 1.1. Examples of *E. coli* genetic functions known to be required for plasmid replication (Scott, 1984; Abeles and Austin, 1987; 1988; Masai and Arai, 1988; Pinkney *et al.*, 1988; Haring and Scherzinger, 1989; Biek and Cohen, 1989)

Plasmid	Host-genes whose products are required
F	<i>dnaA, -B, -C, -E</i>
P1	<i>dnaA, -B, -C, -E, -G, dam</i>
pSC101	<i>dnaA, -B, -C, -G, himA, himD</i>
R1	<i>dnaB, -C, -E, -G, gyr</i>
R6K	<i>dnaA, rpo</i>
RK2	<i>dnaA, -B, -E, -G</i>
RSF1010	<i>dnaZ, gyr</i>
pSa	<i>dnaB, -E</i>
ColE1	<i>dnaB, -C, -G, -Z, polA, rpo</i>

Table 1.2. Summary of the Rep^a proteins of various plasmids

Plasmid	Designation of the Rep protein	Molecular weight
F	RepE	29,000
ColV-K30	RepI	39,000
P1	RepA	32,000
Rts1	RepA	33,000
pSC101	Rep101	37,500
R6K	π	35,000
pSa	RepA	35,000
RK2	TrfA ^b	32,000
RSF1010	RepA	29,000
	RepB'	36,000
	RepC	31,000
pTAR	RepA	28,000

^aRep protein is the plasmid specified protein that is absolutely required for replication to be initiated from the respective plasmid's origin(s) of replication.

^bOnly the MW 32,000 protein that is sufficient for replication in *E. coli* is shown here. A larger protein (MW 43,000) synthesized from a translational start site upstream of the MW 32,000 protein is required for replication in *P. aeruginosa* (Durland and Helinski, 1987).

'RepB' is part of the larger RepB protein (MW 70,000), yet is sufficient for replication for RSF1010-based miniplasmids (Haring and Scherzinger, 1989)

1.2.1. F

The classical sex-factor F is among the earliest and well described extrachromosomal genetic elements and belongs to the IncFI group. *In vitro*, *EcoRI* digestion of the 94.5 kb F plasmid yielded 19 restriction fragments and two of these, mapped on the F plasmid as f5 and f7 (nucleotide co-ordinates 40.3-49.3 and 33.0-40.3 respectively on F). Both these fragments can apparently act as autonomous replicons *in vivo* (Lovett and Helinski, 1976; Manis and Kline, 1977; 1978). The genetic functions of the replicon of the f5 fragment are similar to that of the wild type F and hence, f5 has been intensely investigated and is described in detail here. The replicon contained in f7 is unstable and apparently plays a less significant role in the normal replication of the F plasmid.

At least two origins of replication, *oriV* and *oriS* have been localized in fragment f5 (Fig. 1.3). Replication from *oriV* proceeds in a bidirectional fashion (Eichenlaub *et al.*, 1977) and that from *oriS* is unidirectional (Figurski *et al.*, 1978; Manis and Kline, 1978). All other DNA segments except 44.95-46.00 (a subset of f5; Fig. 1.3) are dispensable for the maintenance of f5 (Lane, 1981). This region includes *oriS* and the *repE* gene. *repE* encodes a protein of MW 29,000 (Watson *et al.*, 1982; Table 1.2). Two sets of directly repeated DNA sequences have been identified on either side of the *repE* gene (see Fig. 1.3). A cluster of four repeats is present within *oriS* and a second cluster of five repeats constitute the *incC-cop* region (Murotsu *et al.*, 1981, Fig. 1.3). The *incC-cop* region is involved in the expression of plasmid incompatibility and maintenance of plasmid copy number. Partial repeats of this sequence are also present within the promoter region of *repE*. Two putative DnaA protein binding sites have been identified within *oriS* and the deletion of these sites abolishes origin activity (Murakami *et al.*, 1987) signifying their importance in F replication.

Fig. 1.3. Structural features of the basic replicons of plasmids F (only the features of the f5 fragment are shown) (Kline, 1988; Yarmolinsky and Sternberg, 1988) and pColV-K30 (Perez-Casal *et al.*, 1989). Note that in Figs. 1.3-1.6 only the direct iterons within the replicon are shown. In these figures similar arrowheads are used to represent the direct iterons of the different plasmids. This does not necessarily indicate that the DNA sequences among them are homologous. However, they are internally consistent for any given plasmid.

The restriction enzyme site for *EcoRI* is depicted as *E. r. nC*. *repE* and *repI* indicate structural genes for the replication genes respectively. *oriV* and *oriS* in F indicate *origin* of (primary) vegetative replication and *origin* of (secondary) replication. *ori* in ColV-K30 shows the region containing the plasmid's *origin* of replication. *ccd* contains genes *A* and *B* that couple the plasmid replication and partition to the host-cell's division cycle. *P* indicates the location of the promoter of the structural gene of replication. The different sizes of open and closed arrowheads represent the different kinds of direct iterons. Arrows indicate the location of the *dnaA* boxes and closed circles indicate the location of the GATC sequences within the replicon. The dashed lines in *repI* indicate that the region is not drawn to scale relative to the other regions of the replicon shown.

F

pColV-K30

DnaA protein is an initiator protein of replication for *E. coli oriC* (Kornberg *et al.*, 1988) and has been shown to bind to the consensus nine bp sequence 5'-TTATC/ACAC/AA-3', termed the *dnaA* box (Fuller *et al.*, 1984).

The *oriV* of f5 defined by co-ordinates 42.81-43.01 (Fig. 1.3) is more complex and requires participation of *repC* (Tanimoto and Iino, 1984), *ccdB*, *repE* (Lane *et al.*, 1984) and possibly *repD* (Tanimoto and Iino, 1985; Lane *et al.*, 1986). The *rep* genes encode replication proteins. *ccd* genes *A* and *B* prevent host cell division until plasmid replication is completed thus ensuring stable inheritance of the plasmid (Ogura and Hiraga, 1983). The interactions of these domains (*repC*, *ccdB*, *repE* and *repD*) with *oriV* have not been characterized. A cluster of eight repeats between the operator of *repC* and *oriV* and one within *oriV* are known to be present (Caughey *et al.*, 1986). Two 5'-GATC-3' DNA sequences, one within a 90% A-T rich region of *oriS* and one just before the translational start site of *repE* were found. The N⁶ position of the adenine residue within the sequence 'GATC' is a substrate for methylation by the *E. coli* Dam methylase system (Geier and Modrich, 1979). A probe containing the f5 fragment hybridized to the other IncFI plasmids R453, R386, ColV-K30 and CFA/I+ST⁺::Tn5 (Bergquist *et al.*, 1986) suggesting that the structural and therefore, possibly, the functional features are conserved among these plasmids.

The replication determinants of another well characterized IncFI group plasmid, ColV-K30, are contained within a 1.8 kb segment of plasmid DNA (Perez-Casal and Crosa, 1987). The highlighting features of this region are the presence of six tandem 10 bp direct repeats and six nearly tandem 18 bp repeats. There are also six 'GATC' sequences and two *dnaA* boxes (Fig. 1.3). *repI* is the structural gene for the replication protein and encodes a protein of apparent MW 39,000 (Table 1.2). *repI* is flanked by the repeated segments of

DNA mentioned above and a heterologous cluster of inverted repeats. The second cluster of iterons include *incE*, a region that confers plasmid incompatibility (Perez-Casal *et al.* 1989).

1.2.2. P1

At least two replicons have been identified within the 100 kb prophage P1 (Sternberg and Austin, 1983) which is a plasmid that belongs to the group IncY. The two replicons have been designated R and L and are separated by about 10 kb. DNA sequences contained in the R replicon confer the ability of plasmid mode of existence (the prophage state) to P1 and have been well studied. The minimal determinants of P1 replication are contained within approximately 2 kb (the R replicon). The *cis*- and *trans*-acting determinants of the replicon have been characterized by deletion and subcloning analysis (Austin *et al.*, 1984). The *cis*-acting region includes a region of about 250 bp that specifies origin function. The 2 kb region also contains a structural gene for replication protein (*repA*) that is *trans*-acting. A locus for control of plasmid copy number, *incA* is also located adjacently to *repA* within this 2 kb region (Austin *et al.*, 1982; Abeles *et al.*, 1984). RepA can function *in trans* and is a protein of MW 32,000 (Chattoraj *et al.*, 1985b, Table 1.2). The *ori* contains five direct repeats (Fig. 1.4). Nine repeats of similar DNA sequence are also present within *incA* (Abeles *et al.*, 1984). Five *dnaA* boxes and five GATC sequences have also been recognized within the P1 minimal replicon (Yarmolinsky and Sternberg, 1988).

Fig. 1.4. Structural features of the R replicon of P1 (Yarmolinsky and Sternberg, 1988), Rts1 (Kamio *et al.*, 1984; Yarmolinsky and Sternberg, 1988) and pSC101 (Churchward *et al.*, 1983; Armstrong *et al.*, 1984; Yarmolinsky and Sternberg, 1988).

oriR represents the region containing the *origin* of replication of the R replicon of P1. *repA* and *rep101* indicate the structural genes of replication in the respective plasmids. The other symbols are as described in Fig. 1.3.

P1

Rts1

pSC101

1.2.3. Rts1

In the IncT plasmid Rts1, a 1.8 kb plasmid region contains genetic functions for autonomous replication (Itoh *et al.*, 1982). The DNA sequence of this region revealed eight direct repeats in two clusters of five (24 bp) and three (21 bp). These flank the structural gene for the replication protein, *repA* (Fig. 1.4). RepA is a protein of MW 33,000 (Kamio *et al.*, 1984; 1988; Table 1.2). There are also five sets of indirect repeats, three putative DnaA binding sites and four 'GATC' sequences within the 1.8 kb region. DNA sequences at nucleotide co-ordinates 1441-1194 (as described in the DNA sequence of the 1.8 kb mini-Rts1 genome, Kamio *et al.*, 1984) containing the *dnaA* boxes and sites for *E. coli dam* methylation specify the plasmid's origin of replication (Itoh *et al.*, 1987). Interestingly, the consensus DNA sequence of the direct repeats of the Rts1 replicon is also found in the direct repeats within the *ori* of P1 (Kamio *et al.*, 1984). It is not known whether these DNA sequences are interchangeable between these replicons without affecting replication functions.

The replicon of another IncT plasmid, R401 is nearly identical to that of Rts1 (Tabuchi, 1985), except for eleven nucleotides in the C-terminal end of the *repA* gene.

1.2.4. pSC101

The origin of replication of pSC101 was identified to be present in a 1.1 kb DNA segment by Tn1000 insertional analysis (Churchward *et al.*, 1983). Replication from pSC101 has been postulated to be driven when the cognate *trans*-acting functions were provided on a heterologous plasmid (Linder *et al.*, 1983). The origin region contains an iteron family and insertion into one of the three 18 bp members of this family abolished plasmid replication suggesting that the iterons perform a critical *cis*-acting role. The effects of insertion in the

other repeats is not known. Three *dnaA* boxes and one 'GATC' sequence were recognized within the pSC101 origin (Fig. 1.4). The structural gene of replication was designated *rep101* and encodes a protein of MW 37,000 (Armstrong *et al.*, 1984; Table 1.2).

1.2.5. R6K

The presence of multiple origins of replication is also a feature of the 38 kb conjugative IncX group plasmid R6K. Three origins α , β and γ have been isolated from R6K (Crosa, 1980). All of these are contained within a 3.9 kb region of the plasmid (Fig. 1.5). Replication from the α and β origins is bidirectional and proceeds towards a well defined termination site on the plasmid genome (Bastia *et al.*, 1981). *In vivo*, the α origin is used predominantly (Crosa, 1980). The minimal regions capable of replication encompass *ori- γ* and the structural gene for replication *pir* (protein for initiation of replication), encoding a basic protein π of MW 35,000 (Inuzuka and Helinski, 1978; Germino and Bastia, 1982; Filutowicz *et al.*, 1985a; Table 1.3). The γ origin contains seven 22 bp direct tandem iterons. An eighth copy of this repeat is present in the operator-promoter region of the *pir* gene (Stalker *et al.*, 1982; Fig. 1.5). A minimal origin region has been localized to 260 bp (Filutowicz *et al.*, 1985a). Loss of one repeat within *ori- γ* does not affect origin capabilities, but loss of three or more repeats abolishes plasmid replication (Kolter and Helinski, 1982) suggesting a minimum requirement of the iteron sequences for *cis*-acting functions. Also *ori- γ* is required *in cis* for replication to proceed from either *ori α* or *ori β* (Shaffermann *et al.*, 1981). In another IncX group plasmid, R485, a 1.4 kb *HindIII*-*BglII* restriction fragment has been found to contain recognition functions for origin activity (Stalker and Helinski, 1985). The salient features of this sequence include the presence of six 22 bp tandem repeats and eight 'GATC' sequences. An adjacent 3.2 kb *BglII* fragment encodes two proteins of MW

Fig. 1.5. Structural features of the replicons of plasmids R6K (Filutowicz *et al.*, 1985a) and pSa (Tait *et al.*, 1983)

Three origins of replication, *ori α* , *ori β* and *ori γ* are shown in the R6K system. *pir* represents the gene that encodes the protein for initiation of replication, the π protein. *repA* is the structural gene of replication in the pSa replicon. The other symbols are as in Fig. 1.3.

R6K **pSa**

18,000 and 14,000 respectively in *E. coli* mini-cell assays (Stalker and Helinski, 1985) and may contribute to the *trans*-acting function(s). No genetic complementation between the π protein of R6K and the origin segment of R485 and conversely, the 3.2 kb *Bgl*II DNA fragment of R485 and the three origins of R6K was observed indicating that although both R485 and R6K belong to the same Inc group, they have diverged considerably and probably adopted different genetic functions for DNA replication. Interestingly, the π protein of R6K can partially complement mutations in the replication protein RepA of plasmid pSa, an IncW group plasmid (Tait *et al.*, 1983).

1.2.6. pSa

pSa is a broad-host-range 38 kb IncW group plasmid encoding resistance to the antibiotics chloramphenicol, kanamycin and spectinomycin (for a recent review of IncW group plasmids see Valentine and Kado, 1989). The minimal plasmid determined regions for autonomous replication of pSa and another IncW plasmid, R388 have been localized to within 4 kb and approximately 5.5 kb plasmid regions respectively. The origin of replication of pSa has been mapped to an approximately 0.47 kb fragment of DNA (Tait *et al.*, 1982). The origin presents two direct repeats of 13 bp each (Fig. 1.5). The structural protein for replication is a polypeptide of MW 35,000 (Table 1.2) encoded by *repA* which apparently acts on the origin *in trans*.

1.2.7. RK2

RK2 belongs to the IncP group and is similar or identical to RP4, R18 and R68 (Burkardt *et al.*, 1979). The parental plasmid is approximately 60 kb in size and encodes resistance to ampicillin, kanamycin and tetracycline. RK2 is maintained in a wide variety of

Gram-negative bacteria (Schmidhauser and Helinski, 1985). Several reviews have been published on the vegetative replication, conjugative transfer functions and broad-host-range ability of RK2 (Figurski *et al.*, 1985; Thomas *et al.*, 1985; Thomas and Smith, 1987; Thomas and Helinski, 1989; Guiney and Lanka, 1989). Figurski and Helinski (1979) demonstrated that replication of a miniplasmid containing the presumptive origin of RK2 is dependent on a plasmid encoded function which could be provided *in trans*. The minimal DNA segment essential *in cis* to provide origin function (*oriV*) is about 400 bp (Stalker *et al.*, 1981b; Cross *et al.*, 1986). Replication from this unique *ori* proceeds unidirectionally as shown by electron microscopic studies on replication intermediates by Meyer and Helinski (1977). Additional DNA sequences on either side of this minimal *ori* modulate *oriV* activity. *oriV* contains a family of five 17 bp iterons. Also four direct repeats (similar to the iterons within *oriV*), two of which are in tandem are found on one side of *oriV* and a set of indirect repeats on the other (Stalker *et al.*, 1981b). Two putative DnaA binding sites flank the five tandem iterons and one of them is contained within an A-T rich region (Fig. 1.6). The salient features of *oriV* are also conserved in a closely related plasmid R751 (Pansegrau and Lanka, 1987; Thomas and Helinski, 1989). Whereas a 393 *Hpa*II fragment containing the *oriV* of RK2 is sufficient to specify origin functions in *E. coli* and *Pseudomonas putida*, a region smaller than this exhibits apparent origin function in *Pseudomonas aeruginosa* (Cross *et al.*, 1986; Nash and Krishnapillai, 1987). Thus, host-specific origin function seems to be a characteristic feature of RK2. In addition to *oriV*, a structural gene, *trfA*, is indispensable for RK2 replication (Table 1.2). The *trfA* gene is part of the *trfA* operon and specifies two proteins of MW 32,000 and 43,000 respectively with alternative translational start sites within the same ORF (Kornacki *et al.*, 1984; Shingler and Thomas, 1984; Smith and Thomas, 1984).

Fig. 1.6. Structural features of the replicons of plasmids RK2 (Thomas and Helinski, 1989), RSF1010 (Haring and Scherzinger, 1989) and pTAR (Gallie and Kado, 1988)

oriV represents the *origin* of vegetative replication. The region *oriV* shown here for the RK2 system is the minimal origin region as described in *E. coli* and *Pseudomonas putida*. A smaller region that includes this origin is apparently sufficient for a functional *oriV* in *P. aeruginosa*. *trfA* indicates the structural gene of replication in RK2 (*trans*-acting factor). *repA*, *repB* and *repC* are structural genes of replication in RSF1010 and pTAR. The other symbols are as denoted in Fig. 1.3.

RK2 **RSF1010** **pTAR**

Both are predictably basic proteins. Other genes in RK2 with a role in plasmid replication are *trfB* and multiple sets of *kil-kor* genes. A *kil* gene specifies host- or plasmid-lethality in the absence of its cognate *kor* gene in the same cell. In other words, the *kor* genes override the function of the *kil* genes to maintain viability of the plasmid or the cell.

1.2.8. RSF1010

Plasmids of the IncQ group also exhibit a broad host-range (Frey and Bagdasarian, 1989). They are relatively small mobilizable plasmids but are not self-transmissible. They carry a *cis*-acting site, *oriT* and *mobilization* genes for self-mobilization in bacterial matings when the transfer functions of some other plasmids (e.g. IncI, IncM, IncX and IncP plasmids) are provided by a 'helper' plasmid co-resident in the cell (Willett and Crowther, 1981). RSF1010, R1162 and R300B are the best characterized IncQ plasmids. Comparison of the restriction enzyme cleavage maps and the location of antibiotic genes and genetic functions for plasmid replication and mobilization on the plasmid genome of these three plasmids suggests that they are identical or similar plasmids. A 2.1 kb DNA fragment of the 8.7 kb RSF1010 plasmid was shown to contain origin function when the 4.2-8.7 kb region of the plasmid region (as described in the restriction map of RSF1010 genome) was provided *in trans*. This was named *oriV*. Location of *oriV* on this 2.1 kb segment was further confirmed by electron microscopic studies on replication intermediates. Subcloning of this 2.1 kb DNA showed that a functional *oriV* is contained within 395 bp and that replication proceeds bidirectionally (Scholz *et al.*, 1985). Four 20 bp direct repeats (Fig. 1.6), contained within the minimal *oriV* have been identified. A 152 bp region with dyad symmetry separated by a 174 bp region containing a 28 bp G-C rich DNA segment followed by a 31 bp A-T rich DNA segment was also identified (Haring and Scherzinger, 1989). The orientation of

the 152+174 bp region with respect to the direct repeats was shown not to be critical for *oriV* activity. Also, small insertions or deletions do not perturb origin function. A similar organization of the origin region was observed in plasmid R1162 (Meyer *et al.*, 1985). The direct repeats of R1162 have been shown to play a role in the expression of plasmid incompatibility and maintenance of plasmid copy number (Lin and Meyer, 1986; Lin *et al.*, 1987). Three structural genes *repA*, *repB* and *repC* were identified in the RSF1010 system as contributing to the autonomous replication abilities of the plasmid (Scherzinger *et al.*, 1984). RepB' is a protein of MW 36,000 and is part of a larger RepB (MW 70,000) protein translated in the same reading frame. The deletion of DNA sequences to eliminate RepB but not RepB' does not affect plasmid replication (Haring and Scherzinger, 1989). RepA is a protein of MW 30,000 and RepC of 28,000 (Table 1.2). These proteins have been purified from *E. coli* cells by specific expression systems and their properties have been studied *in vitro* (Haring and Scherzinger, 1989). *repA* and *repC* are located adjacently to each other in the RSF1010 genome and separated from *repB* (Fig. 1.6). In R1162, *repI* and *repII* are the two structural units of replication in addition to *oriV* (Meyer *et al.*, 1985). Their map locations in R1162 correspond to that of *repAC* and *repB* in the RSF1010 map (Fig. 1.6). *repI* encodes two polypeptides of MW 29,000 and 31,000 respectively (Kim and Meyer, 1985) and may be comparable to the RepAC system of RSF1010.

1.2.9. R1

Plasmids R1, NR1 (also referred to as R100 or 222) and R6-5 are among the well-characterized plasmids of the IncFII group (reviewed by Womble and Rownd, 1988). Extensive studies on NR1 and R1 have shown that the basic features of the replicon are conserved among these plasmids. Hence, published information on R1 and NR1 are used

interchangeably here. R1 is a 94.5 kb conjugative, multiple antibiotic resistance plasmid and all replication determinants are located in a 2.6 kb DNA fragment within nucleotide coordinates 88-91 kb on the R1 map (Molin *et al.*, 1979; Miki *et al.*, 1980; Nordstrom *et al.*, 1984). This includes an *ori* containing segment adjacent to which is the structural gene, *repA1*, that encodes a *cis*-acting protein (Masai and Arai, 1988) of MW 33,000 (Rosen *et al.*, 1980; Armstrong *et al.*, 1986; Table 1.2). Three additional structural genes *repA2* (*copB*), *repA3* and *repA4* have also been identified (Womble and Rownd, 1988). *repA2* and *repA3* are upstream of and *repA4* is downstream of *repA1*. Transcription of *repA1* is (a) constitutive from a weak promoter upstream of *repA2* and (b) regulated from a moderately strong promoter downstream of *repA2* (Fig. 1.7). *repA1* and *repA2* are in the same transcriptional unit when expressed from the weak promoter. Expression of *repA1* from the strong promoter is repressed by the translated product of *repA2* (Light and Molin, 1981; 1982). A 93 nucleotide long *incA* RNA (from the *copA* gene) read in the opposite orientation as that of *repA1* and complementary to the 5' domain of the *repA1* mRNA is also a significant feature of the NR1 replicon (Rosen *et al.*, 1981; Stougaard *et al.*, 1981; Fig. 1.7). *incA* and *repA2* are also named *cop* genes because mutations within these genes result in changes in plasmid copy number (Nordstrom and Nordstrom, 1985). The precise genetic functions of *repA3* and *repA4* are currently unknown and they are therefore not shown in Fig. 1.7. Structural analysis of the *inc* RNA (also called *copA* RNA) has revealed two stem-loop structures for the molecule connected to each other by a spacer region and a single-stranded 3' tail (Wagner and Nordstrom, 1986). LoopII of the stem-loop structures is accessible for base pairing with the *repA1* mRNA.

Fig. 1.7. Structural and functional organization of the replicon of plasmid R1. *ori* indicates the region wherefrom plasmid DNA replication is initiated. *repA1* is the structural gene of replication and encodes a protein RepA1 (MW 33,000) that acts *in cis* on the *ori* to initiate replication (+). The mRNA encoded from the *repA1* gene is called RNA A or RNA II. *repA2* (*copB*) encodes a small protein (MW 11,300) that binds to the promoter (P2, a moderately strong promoter) of *repA1* and represses its transcription (indicated as (-)). RepA2 can act *in trans*. P1 is the promoter for *repA2*. It is a weak promoter and expression from it is constitutive. Transcription from this promoter produces a long RNA molecule (RNA-CX) from which both RepA2 and RepA1 could be translated. *incA* RNA is encoded from *copA* in an opposite orientation to that of *repA2* and *repA1* from promoter P3 and is complementary to the mRNA of *repA1*. This leads to hybridization between these two kinds of RNAs which leads to an inhibition of *repA1* mRNA translation. Thus, *incA* RNA regulates expression of *repA1* post-transcriptionally. The *incA* RNA has also been called RNA E. (Scott, 1984; Rownd *et al.*, 1985; Womble and Rownd, 1988; figure adapted from Novick, 1987).

R1

Although small inverted repeat structures were detected within the *ori* of R1 (Ryder *et al.*, 1981), direct iterons of the kind discovered in other plasmids (e.g. pCU1, F, P1, Rts1, RK2, RSF1010, etc.) are absent (Masai and Arai, 1988).

The DNA sequence of the RepI origin region of another IncFII plasmid, ColV-K94 has been established. Extensive similarities between the origin regions of R1 and ColV-K94 were observed (Weber, 1985).

1.2.10. ColE1

Involvement of RNA molecules in plasmid replication is also a characteristic feature of this group of plasmids which includes ColE1, pMB1, p15A and RSF1030 (Selzer *et al.*, 1983). The ColE1 replicon does not require plasmid-determined proteins for replication. Also, unlike the several systems described above, no remarkable structural features such as the direct iterons are noticeable in the ColE1 *ori*. However, the tetranucleotide 'GATC' and *dnaA* boxes are found in pBR322 (Kline *et al.*, 1986; Russell and Zinder, 1987). The ColE1 group thus, probably represents the simplest form of plasmid replicon organization. The precise *ori* of ColE1 has been mapped to any of the three successive AAC residues contained within the origin containing fragment (Tomizawa *et al.*, 1977). The nucleotide sequence of the replication regions of plasmids ColA and ColD show extensive homology to that of ColE1 which suggests that the organization and the mode of replication of these plasmids are identical (Zverev and Khme', 1985).

In the closely related colicinogenic plasmids, ColE2 and ColE3 a *trans*-acting structural gene of replication has been mapped (Horii and Itoh, 1988). This *rep* gene is essential for the autonomous replication of these plasmids. The *rep* genes of ColE2 and ColE3 are >90% homologous to each other. The *ori* of ColE2 has been mapped to a

contiguous 32 bp DNA segment and that of ColE3 to a 33 bp DNA segment (Yasueda *et al.*, 1989). Synthesis of an untranslated RNA molecule, *incA* RNA (similar to the *inc* RNA of plasmid R1), complementary to the Rep mRNA and the possible control it exercises on its translation has also been raised by the same authors. Notably, long direct or indirect repeat sequences are absent within the 1.5 kb autonomously replicating segments of ColE2 and ColE3. However, two smaller direct repeats of 7 bp and 9 bp are found in both these plasmids and are separated by dissimilar spacer regions.

1.2.11. Other plasmids

The other plasmids isolated from Gram-negative bacteria whose structural features have been characterized include pTAR and pRiA 4b (isolated from *Agrobacterium* sp.), pJD4 (from *Neisseria* sp.) and pTF-FC2 (from *Thiobacillus* sp.). The replication functions of the 44 kb pTAR are located on a 1.3 kb *EcoRV*-*AvaII* restriction fragment (Gallie and Kado, 1988). A single origin has been mapped by electron microscopy. A structural gene *repA* that encodes a replication protein of MW 28,000 (Table 1.2) has also been identified. Two clusters of 8 bp repeats flanking *repA* are found (Fig. 1.6). On the 5' end of *repA*, the cluster contains four and at the 3' end three additional direct repeats. The 1.3 kb fragment also contains loci encoding functions for stable inheritance of the plasmid and for determination of plasmid copy number (Gallie and Kado, 1987). A putative DnaA binding site can also be recognized at nucleotide positions 401-409 in the origin containing pTAR fragment (see Gallie and Kado, 1988).

Plasmid pTF-TC2 is a 12.4 kb plasmid isolated from *Thiobacillus ferrooxidans* and its replicon is functional in *E. coli*. The minimal replication region as defined in *E. coli* was shown to be contained in a 2 kb DNA fragment (Dorrington and Rawlings, 1989). Origin

and incompatibility determinants are expressed from a 184 bp DNA region of the 2 kb. Three tandem iterons of 22 bp each and two 'GATC' sequences can be found. Multiple sets of direct repeats, two *dnaA* boxes and at least three 'GATC' sequences are found within a 1.5 kb region specifying the replication units of the 7.3 kb penicillinase plasmid, pJD4 (Yeung and Dillon, 1988).

A 4.6 kb region of the 250 kb hairy-root inducing plasmid pRiA 4b of the *Agrobacterium* sp. was shown to contain all determinants for plasmid replication (Nishiguchi *et al.*, 1985). The features of this replicon include the presence of three structural genes of replication *repA*, *repB* and *repC* encoding putative polypeptides of MW 45,000, 34,600 and 44,300 respectively. A 26 bp palindromic sequence was also identified within the replicon. However, direct iterons of the kind present in plasmids F, P1 and Rts1 were not recognized.

From the above review it can be seen that the known plasmid replicons can be grouped into three different classes. Class I includes ColE1-type replicons that do not contain genes specifying proteins essential for plasmid DNA replication. Iterons are also absent in them. Close relatives of this group are ColE2 and ColE3 that encode a Rep protein as part of their replication machinery yet lack well-defined iterons. Class II is exemplified by F, P1, and Rts1. In these plasmids, the *rep* gene is flanked by two sets of repeats that may (e.g. F and P1) or may not (e.g. ColV-K30) be similar to each other. In the replicons of pSC101, R6K, R485, RK2, RSF1010 and pTF-FC2 that comprise Class III, the iterons do not flank the *rep* gene(s). A common feature of the latter two groups is the presence of a structural *rep* gene(s) encoding a replication protein. It should be noted however, that although *oriV* of RK2 contains five tandem iterons, homologous iterons are dispersed on either side of *oriV* (Fig. 1.6). Also, in R6K an eighth member of the iteron family is located distantly from the other group of seven tandem iterons. Most of the

plasmid replicons of all three classes contain *dnaA* boxes and *dam* methylation sites. This indicates that irrespective of the divergence in plasmid evolution, certain common features in the utilization of the host-encoded factors for plasmid replication are conserved.

1.3. MOLECULAR MECHANISMS OF PLASMID REPLICATION

1.3.1. Models for the regulation of plasmid replication

Plasmids are maintained at a certain average copy number within a population of bacteria under specific growth conditions (for a recent review on mechanisms of plasmid copy number control see Thomas, 1988). Plasmids such as F, P1 and NR1 are maintained at a copy number of 1 or 2 and are designated as 'low' copy number plasmids. RK2, R6K and RSF1010 are 'moderate' copy plasmids and are maintained at about 5-10 copies per cell. ColE1, ColDF13 and pMB1 are 'high' copy number plasmids (about 15-20 copies per cell). DNA replication is believed to be controlled at the level of initiation in order to maintain a plasmid at a copy number that is characteristic of the system. Two models have been proposed to explain the regulation of plasmid replication (Jacob *et al.*, 1963, Pritchard *et al.*, 1969; Sompyrac and Maaloe, 1973). The 'replicon' model of Jacob *et al.*, postulates a structural gene, that encodes an 'initiator' molecule and a site of its recognition, the 'replicator' upon which the initiator acts to initiate replication. The authors further suggested that specific sites are present on the replicon for attachment to the bacterial membrane surface. This enables the daughter molecules to be faithfully distributed to the daughter cells following replication and cell division. However, the model does not accommodate mechanisms for the control of plasmid copy number. In the 'inhibitor-dilution' model of Pritchard *et al.* (1969), replication is negatively regulated by a 'repressor' molecule. As in the 'replicon' hypothesis, the 'inhibitor-dilution' model predicts a constitutively

synthesized 'initiator'. Furthermore, Pritchard *et al.* proposed that an 'inhibitor' is synthesized from a plasmid region located adjacent to the replicon and is made only when replication is initiated or is unstable. The inhibitor then prevents a second round of replication by either interacting with the origin, making it unavailable for initiator action or by inhibiting expression of the initiator itself. In the former case, the inhibitor could alter the higher order topology of the origin region or simply block access of the origin to the initiator as a result of formation of the origin-inhibitor complex. In a single round of replication, sufficient amount of inhibitor is synthesized to persist for a cycle of cell growth. With increasing cell volume the concentration of the inhibitor is diluted below a critical level and another round of replication is initiated. The 'autorepressor' model of Sompyrac and Maaloe is similar to the 'inhibitor-dilution' model except that the initiator specifies an autoregulatory function in addition to its initiation function. As will become apparent in the following paragraphs, examples of all three models have been observed.

1.3.2. Regulation of plasmid replication

A review of the available information on plasmid DNA replication suggests that plasmids as a group utilize two different modes of regulating their replication although there may be variations of each mode and a degree of overlap between the different modes. In the first of these modes as exemplified by the replication of ColE1, an RNA molecule has the principal role of determining the initiation of replication. In contrast, in several other plasmids, a Rep protein(s) is an essential determinant of replication initiation.

1.3.2.1. Regulation by Mode I

Plasmids like ColE1, CloDF13, p15A, pMB1 and RSF1030 adopt this mode of replication regulation. The general attributes of these plasmids include (a) requirement of the host-encoded DNA polymerase I protein for replication and (b) no requirement of any plasmid encoded polypeptides for the initiation of plasmid replication and (c) absence of a requirement for *de novo* protein synthesis for plasmid DNA replication (Clewell, 1972); this property was inferred by the ability of ColE1 DNA to replicate *in vitro* when crude extracts from plasmidless *E. coli* cells were provided and the ability of the plasmid to continue replication *in vivo* in the presence of inhibitors of protein synthesis (e.g. chloramphenicol). This phenomenon of continued plasmid DNA replication after arrest of chromosomal replication leads to a dramatic increase in plasmid DNA and has been termed 'amplification'. The functional organization of the ColE1 replicon is well-studied and is presented briefly below.

Replication *in vitro* of the ColE1 replicon is initiated at a unique site called *ori* at any of the three successive AAC residues (Tomizawa *et al.*, 1977). At first, the host-encoded DNA dependent RNA polymerase initiates synthesis of a preprimer RNA (RNA II), from about 555 bp upstream of the *ori* and proceeds towards it. This RNA-DNA hybrid is a substrate for processing by RNase H to generate the primer RNA with a free 3' -OH end (Fig. 1.8). DNA polymerase I begins adding deoxyribonucleotides to this primer and thus, replication is initiated (Itoh and Tomizawa, 1981). The primer RNA is removed by RNase H or by the exonuclease function of DNA polymerase I (Itoh and Tomizawa, 1981). Paradoxically, Ogawa and Okazaki (1984) found that plasmid ColE1 could be maintained in RNase H mutants of *E. coli*. In such cases, a DNA *polA* independent replication is probably adopted by the plasmid (Kogoma, 1984). The ability of RNase H to recognize

Fig. 1.8. Regulation of replication in ColE1. +1 indicates the origin of DNA replication.

Approximately 555 bp upstream of the origin, synthesis of preprimer RNA (RNA II) is initiated from promoter P2 and proceeds towards the origin. RNA II assumes successive secondary structures (hairpin-loop structures) during its synthesis and an early conformation provides complementary sites for hybridization with RNA I (bottom panel). RNA I is synthesized in the opposite direction as that of RNA II from a promoter (P1) about 400 bp upstream of the origin. RNA I also exhibits stem and loop structures. Its hybridization with RNA II allows transcription to proceed through the origin. However, if RNA II escapes hybridization with RNA I, it assumes a later secondary conformation that prevents its accession to RNA I. This results in the formation of a loop (L), that is recognized by RNase H (Rn). RNase H cleaves this mature preprimer RNA (mature RNA II) and generates a free 3'-OH end to which DNA polymerase I begins adding deoxyribonucleotides to initiate DNA replication (Re) (Scott, 1984; Polisky, 1986; 1988; figure adapted from Novick, 1987).

ColE1

specifically and cleave RNA species at the origin of replication can probably be explained by the unique secondary structure of the *ori* region (Hillenbrand and Staudenbauer, 1982; Masukata and Tomizawa, 1984; see below).

Regulation of ColE1 replication is achieved at the level of RNA II-DNA hybrid formation. The main controlling factor is a 108-110 nucleotides long untranslated RNA molecule, RNA I (Levine and Rupp, 1978; Lacatena and Cesareni, 1981; Tomizawa and Itoh, 1981). RNA I is transcribed in a direction opposite to the direction of transcription of RNA II and is initiated approximately 400-480 nucleotides from *ori* (Itoh and Tomizawa, 1981). Therefore, the 5' end of RNA II is complementary to RNA I (see Fig. 1.8). *In vitro*, addition of RNA I inhibits formation of primer and therefore, replication (Tomizawa et al., 1981). This indicates that RNA I can act *in trans* and mediate expression of plasmid incompatibility. Enzymatic and chemical probe analysis have revealed a higher order structure for RNA I (Morita and Oka, 1979; Tamm and Polisky, 1983) *in vitro*. A 5' leading single stranded region and three stem-loop structures are the main features of RNA I topology (see Fig. 1.8). Mutations that potentially affect the secondary structure of RNA I have been obtained (Conrad and Campbell, 1979; Muesing *et al.*, 1981) and these resulted in an elevation of plasmid copy number. This indicates that the secondary structure of RNA I is critical to its negative regulatory role.

RNA II has also been postulated to fold into stem and loop structures (Selzer *et al.*, 1983; Fig. 1.8) and this provides stability to the RNA species itself or to the RNA-DNA hybrid and also serves as a recognition signal for RNase H processing. Regulation of ColE1 replication is achieved by RNA I-RNA II interactions (see Polisky, 1986; 1988). As the primer RNA (RNA II) is being transcribed, it successively assumes different secondary structures. An early conformation provides complementary sites for RNA I hybridization.

This permits continuation of transcription through *ori* and replication is not initiated. However, if RNA II escapes hybridization with RNA I, its conformation is changed and it is now no longer accessible to RNA I (Tomizawa, 1984; Wong and Polisky, 1985). This later secondary structure is a signal for RNase H cleavage that leads to replication. Thus, the kinetics of RNA I-RNA II hybridization determines initiation frequency. A second regulatory element (the *rop* or *rom* gene, repressor of primer or RNA I [one] modulator) that encodes a 63 amino acid acidic-polypeptide (Cesareni *et al.*, 1982) has also been identified. This gene is located about 500 bp downstream of the origin and promotes RNA I-RNA II hybridization *in vitro* (Tomizawa and Som, 1984). The molecular details of this catalysis reaction remain to be understood (Polisky, 1986).

1.3.2.2. Regulation by Mode II

Plasmids that encode their own replication protein(s) that acts as an initiator of replication are included in this group. They can be broadly classified into two subdivisions: (a) plasmids that encode a replication protein, the synthesis of which is regulated by an RNA molecule, e.g. plasmids R1, NR1, R6-5 and (b) plasmids in which the synthesis of the replication protein is autoregulated, e.g. plasmids F, P1, pSC101, etc. The latter subdivision includes plasmid R6K, in which a protein called π acts with the RNA molecules synthesized at the origin that directly act on the origin region.

As described before, the replicon structures of plasmids NR1, R1 and R6-5 are similar and contained within about 2.5 kb of the total plasmid molecule. Replication is initiated from the *ori* and proceeds unidirectionally. Adjacent to the origin is the *repA1* gene that encodes a *cis*-acting polypeptide (Ohtsubo *et al.*, 1977; Masai and Arai, 1988). RepA1 is essential for initiation of replication. Upstream of *repA1* is another gene *repA2* (or *copB*)

that encodes a polypeptide of MW 11,000 (Molin *et al.*, 1981). Mutations in *repA2* and *copA* that increase copy number have been obtained suggesting that they perform a negative regulatory role. *copA* encodes an untranslated RNA molecule (*incA* RNA) that is complementary to the 5' end of *repA1* mRNA (Light and Molin, 1983). Thus, *copA* regulates *repA1* expression post-transcriptionally (Fig. 1.7). This is also the basis of plasmid incompatibility because *copA* provided on a heterologous plasmid can regulate *repA1* expression *in trans* (Rownd *et al.*, 1985). RepA2 inhibits expression of *repA1* at the transcriptional level by blocking the second moderately strong promoter of *repA1* for RNA polymerase accession (Molin *et al.*, 1981; Fig. 1.7). Thus, when the number of plasmid copies falls in a cell, the concentration of RepA2 is below a critical level (because it is proportional to gene dose), *repA1* is derepressed and replication is initiated. At this step, overexpression of *repA1* mRNA is prevented by its titration by the *inc* RNA. The *inc* RNA has been shown to exhibit secondary structures *in vitro* (Nordstrom *et al.*, 1988) similar to the stem-loop structure of RNA I of ColE1. The *inc* RNA hybridizes to the leader sequence of *repA1* mRNA that contains the ribosome binding sites of the molecule (Womble *et al.*, 1984) thus, inhibiting its translation. The precise mechanism of RepA1 action on the *ori* to initiate replication is yet unravelled.

The molecular functions of the replication proteins of RSF1010 have only recently been identified (Haring and Scherzinger, 1989). RepA, RepB' and RepC showed helicase, primase and site-specific DNA binding activities respectively *in vitro*. RepB' is a truncated version of RepB and retains the functions of RepB. Based on the enzymatic properties and electron-microscopic studies on the DNA-protein interactions of RepA, RepB' and RepC on RSF1010 DNA, a model for the initiation of replication of RSF1010 has been proposed

and is briefly described below. RepC recognizes and binds to the four 20 bp repeats within *oriV* (Fig. 1.6) and folds the DNA into looped structures. Since, replication initiation signals are present on both the complementary strands of *oriV* (Honda *et al.*, 1988), 'lagging' strand synthesis is not involved. The looped duplex DNA then unwinds because of the structural changes at *oriV*. RepA now binds to the unwound ssDNA to maintain this conformation and to facilitate entry of RepB'. RepB' then synthesizes the primer molecule that serves as the substrate for elongation by DNA polymerase III and replication is continued on both the strands. RepA moves ahead of the replicating complex to keep the DNA in an unwound state. In the closely-related plasmid R1162, Kim and Meyer (1986) have identified a 75 base long RNA molecule that negatively regulates expression of the *repI* gene at the post-transcriptional level. This RNA is complementary to a segment of the RepI mRNA and thus, could prevent its translation by titrating it by RNA-RNA hybridization.

The mechanisms of Rep protein-*ori* interaction have also been well studied in F, P1, RK2 and R6K. A general scheme of replication regulation in such plasmids is depicted in Fig. 1.9.

In F, protein RepE is *trans*-acting (Tolun and Helinski, 1982; Tokino *et al.*, 1986; Kline, 1983). Deletion of iterons from the right cluster in *oriS* (Fig. 1.3) led to an increase in plasmid copy number (at least 6-10 times that of the wild type state) in mini-F plasmids. To account for this, Tsutsui *et al.* (1983) proposed a 'titration' model in which they suggested that RepE is an activator of replication and is titrated out by binding to the iterons. This prevents excessive initiation of replication and keeps the plasmid copy number in check. RepE also binds to its own promoter within the repeated sequences that are considerably homologous to the two clusters of iterons. This represses *repE* transcription (Sogaard-Andersen *et al.*, 1984; Tokino *et al.*, 1986) and therefore, RepE is autoregulatory.

Fig. 1.9. A general scheme of regulation of replication in plasmids like F, P1, Rts1 and R6K (see Section 1.3.3.2)

ori and *rep* represent the origin and the structural gene of plasmid replication respectively. The major features of the *ori* are the presence of direct iterons, *dnaA* boxes, GATC sequences (symbols as described in Fig. 1.3) and A-T rich DNA segments (not shown here). The *rep* gene encodes a replication protein, Rep (see Table 1.2), that acts positively (+) on the origin by binding to it to stimulate initiation of replication. Rep also acts negatively (-) on the promoter of the *rep* gene by binding to it and repressing transcription from this promoter. Thus, it inhibits synthesis of the Rep protein and is autoregulatory (see also Section 1.3.1).

Hypothetically, RepE has two states, one that acts positively on the *ori* to initiate replication and another that represses *repE* expression at the transcriptional level (Trawick and Kline, 1985).

Binding of the RepA protein of Rts1 to its own operator-promoter region in order to repress *repA* transcription is also mediated by repeated sequences (Kamio *et al.*, 1988). A deletion derivative of mini-Rts1, in which the C-terminal end of the wild type *repA* is deleted such that the last six amino acids of RepA are absent and replaced by three non-native amino acids was incapable of initiating replication from Rts1 *ori*. This led Terawaki *et al.* (1988) to propose that the C terminus end of RepA is essential for its function. Apparently, the autoregulatory efficiency was also reduced suggesting that the domains for autoregulation and replication initiation are closely linked in RepA of Rts1 unlike RepE of F. A point mutation in the midportion of *repA* led to an increase of plasmid copy number (Terawaki and Itoh, 1985). This points out that the central domain of RepA participates in the control of frequency of replication initiation.

DNase I footprinting studies on P1 *ori* with RepA of P1 (Abeles, 1986; Abeles *et al.*, 1989) have demonstrated that RepA binds to three sites on the mini-replicon: (a) the direct repeats contained within *ori*, (b) the *incA* locus and (c) the promoter of *repA*. Binding of *repA* to *ori* is thought to initiate replication because RepA is absolutely required for P1 DNA replication *in vitro* (Abeles and Austin, 1987; Wickner and Chattoraj, 1987). Secondly, *incA* titrates out RepA to serve as the copy number control element. This is evidenced by an increase in mini-P1 plasmid copy number when the *incA* locus is deleted (Chattoraj *et al.*, 1984; Pal *et al.*, 1986). The *repA* promoter lies within the *ori* region and includes the rightmost of the three 19 bp repeats (Chattoraj *et al.*, 1985a). Thus, binding of the RepA

protein to the *repA* promoter may repress its transcription. Indeed, RNA polymerase is actively dislocated from the promoter region of *repA*, following which RepA binds to this region and renders it inaccessible for transcription (Abeles *et al.*, 1989).

Competition of RNA polymerase and the RepA (encoded by the *rep101* or *repA* gene of pSC101) for the promoter (containing an inverted repeat DNA sequence) of *repA* in the replicon of pSC101 also appears to be the mode of autoregulation of *repA* expression (Vocke and Bastia, 1985; Linder *et al.*, 1985). Binding of RepA to the pSC101 *ori* has also been demonstrated (Vocke and Bastia, 1983) and this could be the basis for the initiation of replication.

Unlike the cases described above, a complex circuitry of interaction of genes is involved in the regulation of plasmid RK2 replication. Although the minimal plasmid-determined genetic functions for replication are contained within *oriV* and *trfA*, a second gene *trfB* that regulates expression of the *trfA* operon was discovered (Thomas *et al.*, 1980). Also designated *korA* or *korD* (Figurski *et al.*, 1982), *trfB* controls expression of *kilD* that is part of the *trfA* operon. A network of other *kil-kor* genes (A,B,C,D,E and probably F) are involved in RK2 maintenance (Thomas and Helinski, 1989). While *kilA*, *kilB* and *kilC* encode functions for host-lethality (Figurski *et al.*, 1982), *kilD* inhibits plasmid replication (Smith and Thomas, 1983). The expression of the respective *kil* genes is regulated by their cognate *kor* genes and *kilD* is controlled by *korA* and/or *korB* (Smith *et al.*, 1984; Bechhofer *et al.*, 1986). Additionally, cross regulation of a *kil* gene by another *kor* gene is also known (see Thomas and Smith, 1987). Nevertheless, *trfA* is the essential Rep protein of the RK2 system. It specifies two polypeptides of MW 43,000 and 32,000 respectively. A derivative of RK2 carrying only the gene for MW 32,000 protein can be maintained in *E. coli*, *Rhizobium*

meliloti, *Agrobacterium tumefaciens*, *Azotobacter vinelandii* and *P. putida*. However, this derivative could not be maintained in *P. aeruginosa*. The gene encoding the MW 43,000 protein was also shown to be required for RK2 replicon maintenance in *P. aeruginosa* (Durland and Helinski, 1987). Pinkney *et al.* (1988) have demonstrated that purified TrfA protein binds to the repeats within *oriV* and that *in vitro* mini-RK2 DNA replication requires an exogenous supply of *E. coli* DnaA, DnaB, DnaG, Dna gyrase and DNA polymerase III holoenzyme besides TrfA protein for replication. Thus, a complex of protein molecules participate in RK2 replication. *trfA* expression is not constitutive and transcription from the gene is repressed by a number of other genes e.g. *trfB* and *korF* (see Thomas and Helinski, 1989). The exact details as to how TrfA participates in the initiation of replication remains to be elucidated. Perhaps the basis for the broad-host-range of RK2 is its ability to recognize and utilize the several host-encoded functions for its own replication in different classes of unrelated bacteria.

The π protein of plasmid R6K encoded by the *pir* gene is an essential replication protein for all the three origins of plasmid R6K. π is a basic protein of MW 35,000 and can act *in trans* (Kolter *et al.*, 1978; Filutowicz *et al.*, 1985a). Purified π protein binds to the seven repeats within the γ origin and also to the lone repeat (that is homologous to the seven repeats) and two inverted 9 bp repeats within the operator-promoter of the *pir* gene (Germino and Bastia, 1983; Filutowicz *et al.*, 1985b, c). Thus, in addition to being an initiator protein, π is also autoregulatory (Filutowicz *et al.*, 1985b) similar to RepA of P1 and RepE of F. π protein not only competes with RNA polymerase to bind to the *pir* gene promoter but also displaces it from preformed RNA polymerase-*pir* gene promoter complex (Filutowicz *et al.*, 1985b; Kelley and Bastia, 1985). Binding of π to the γ origin causes

unwinding of the duplex DNA (Mukherjee *et al.*, 1985) and this π -*ori* γ interaction promotes π -*ori* β interaction (Mukherjee *et al.*, 1988). This may explain why *ori* γ is required *in cis* for a functional *ori* β . Recently, Patel and Bastia (1986) showed that a 'silencer' RNA is transcribed from a region adjacent to *ori* γ . The 'silencer' RNA has been postulated to invade *ori* γ to inhibit replication initiation by probably competing out the primer RNA or by altering the higher order structure of *ori* γ . As a corollary, the same authors showed the presence of an 'activator' RNA synthesized from a region complementary to that encoding silencer RNA (Patel and Bastia, 1987). Activator RNA promotes replication initiation from *ori* γ . Control of replication initiation is probably achieved by titration of the activator RNA by the silencer RNA. In fact, the π protein has been shown to enhance the 'activator-silencer' hybridization *in vitro*. Thus, the activator being no longer available, *ori* γ remains in a repressed state. Indeed, at excessive concentrations π protein is refractory for the initiation of replication (Filutowicz *et al.*, 1986). The mode of replication control employed by the R6K system is reminiscent of the mechanism employed by the ColE1 replicon for control of replication initiation. The major difference is that the Rop (or Rom) protein is dispensable for ColE1 replication but π is an essential replication protein of the R6K replicon. The R6K system is also comparable to that of R1. The contrast in this comparison is that while in R6K, the π protein promotes RNA-RNA (activator-silencer) hybridization, such an RNA-RNA (*inc* RNA-mRNA of *repA1*) interaction inhibits translation of *repA1* mRNA, RepA1 being an essential initiator protein of replication in plasmid R1. In the case of R1162 also, the expression of RepI mRNA is inhibited by a small RNA molecule that is complementary to a segment of the RepI mRNA. This inhibition is probably achieved by RNA-RNA hybridization.

1.4. PLASMID PARTITION

The inheritance of plasmid molecules by all daughter cells following cell division is an integral part of stable maintenance of a plasmid. Distribution of plasmid copies to the daughter cells is probably random and easier for a multicopy plasmid by the very virtue of its high copy number. In such cases, e.g., ColE1, site-specific recombination systems have been identified that resolve multimeric plasmids that can accumulate in a growing population, thus increasing the plasmid pool available for distribution to the daughter cells (Austin *et al.*, 1981; Summers and Sherratt, 1984). Low copy plasmids such as F, P1, R1, NR1 and pSC101 have been discovered to specify plasmid borne genetic function(s) for partitioning. A mechanism common to F and R1 is that plasmidless segregants after cell division are killed (Jaffe *et al.*, 1985; Gerdes *et al.*, 1986). This helps in the propagation of only plasmid-carrying cells in the given population.

Two genes have been identified within a DNA segment encoding partition functions in F (*sopA* and *sopB*, Ogura and Hiraga, 1983). These act on the *cis*-acting *par* site which contains twelve 43-bp direct repeats and a 7-bp inverted repeat within each of the 43-bp units (Helsberg and Eichenlaub, 1985; Mori *et al.*, 1986; Lane *et al.*, 1987). In P1, the minimal *par* site has been shown to be within 29 bp when the structural genes *parA* and *parB* are provided *in trans* (Martin *et al.*, 1987). The overall structural organization of the *par* regions of F and P1 are remarkably similar. Both the *par* systems have also been shown to stabilize heterologous unstable plasmids (Austin and Abeles, 1983; Austin *et al.*, 1984). This demonstrates that the partition mechanism of a plasmid probably functions independent of the minimal replication determinants.

The stability (*stb*) region of NR1 (Tabuchi *et al.*, 1988) and *parA* of R1 (Gerdes and Molin, 1986) exhibit a different organization from that of F and P1 but are homologous to each other and are contained within a 1.5 kb DNA region of the plasmid. Two ORFs, *stbA* and *stbB* encoding putative proteins of MW 36,000 and 13,000 respectively have been identified in the *stb* region of NR1 and encode *trans*-acting proteins that are essential for partition. The NR1 *par* locus is within about 600 bp and is contained partially within the promoter and partially within the 5' domain of the *stbA* ORF. The *parB* system of R1 encodes two genes *hok* and *sok* (Gerdes *et al.*, 1986). *hok* encodes a diffusible product and is regulated at the translational level by *sok*. While *hok* specifies host-lethality and is stable, the *sok* product is unstable. Thus, a cell carrying the *parB* of R1 survives *hok*-induced killing when *sok* is co-resident in the cell. However, if the cell loses the *sok* function by plasmid loss, the blocking function on the *hok* product is relieved and the cell is 'killed'. This leads to the elimination of plasmid-free segregants. An analogous function specified by the *ccdA* and *ccdB* genes has been reported in the F maintenance system (Jaffe *et al.*, 1985).

1.5. PLASMID INCOMPATIBILITY

The utilization of diffusible gene products for plasmid replication and partitioning could lead to a competition between homologous plasmid molecules for a limited supply of gene products. This results in the expression of 'incompatibility' between the plasmids in question. Novick (1987) has described (a) symmetric incompatibility as when either plasmid is lost with an equal probability and (b) vectorial incompatibility as when one plasmid is lost exclusively or with a higher probability than the other.

Symmetric incompatibility occurs when the two plasmids share essential replication and stable maintenance functions. Vectorial incompatibility is expressed because of the interference with the normal replication and partitioning mechanisms by cloned plasmid DNA fragments that contain elements of replication and partition control.

RNA I is an inhibitor of replication in ColE1 (Tomizawa *et al.*, 1981). An exogenous addition of RNA I will therefore, further repress replication. This occurs by the titration of the preprimer RNA (RNA II, the target) by RNA I and replication of ColE1 is blocked. Thus, RNA I mediates expression of incompatibility in ColE1. A similar but slightly varied case is presented by R1 and NR1. The target of *copA* (or *inc*) RNA in these plasmids is the mRNA of *repA1*, a gene coding for an essential replication protein i.e., *inc* RNA and *repA1* mRNA are countertranscripts (Scott, 1984; Rownd *et al.*, 1985). Since the *inc* RNA can act *in trans*, it can inhibit expression of *repA1* and thus, encode incompatibility functions. A secondary mode of incompatibility expression in ColE1 and NR1 is mediated by small protein molecules (Rom and RepA2 respectively). Their mode of replication regulation was described before.

The iterons within or near the replication origins are the major sites of incompatibility expression in plasmids like F, P1, etc. In these plasmids, replication proteins are activators of DNA replication and they bind to the iterons in the replication region (Bastia *et al.*, 1985; Filutowicz *et al.*, 1985c; Pinkney *et al.*, 1988; Abeles *et al.*, 1989). Thus, titration of the replication protein is a major regulatory circuit of replication control and hence, expression of plasmid incompatibility (Tolun and Helinski, 1981; Kline and Trawick, 1983). In other words, deletion of the iterons results in the loss of plasmid incompatibility and also to an increase in plasmid copy number (because replication is deregulated). This has been demonstrated for the F plasmid (Tsutsui *et al.*, 1983).

All of the above are examples of replication associated incompatibility expression. Partition associated incompatibility expression has also been documented in plasmids F, P1 and R1 (Nordstrom *et al.*, 1980; Austin and Abeles, 1985). As stated in the previous section, the *par* regions contain a *cis*-acting *par* locus and *trans*-acting *par* genes. The product of the *par* genes act by binding to the *par* locus. Since the *par* genes do not exhibit replicon specificity, they can bind to the *par* loci, even when the latter are present on heterologous plasmids and hence, cause incompatibility (Austin and Abeles, 1985).

As stated earlier, the subject of this dissertation is the IncN replicon of plasmid pCU1. Following a description of the materials and methods used (Chapter 2), the results and discussion are organized in three sections, which consider in turn, the host-range (Chapter 3), structural organization (Chapter 4) and functional organization (Chapter 5) of the replicon of pCU1.

CHAPTER 2

Materials and Methods

2.1. BACTERIAL STRAINS, PLASMIDS AND BACTERIOPHAGES

Tables 2.1-2.4 list the bacteria, plasmids and bacteriophages used in this study.

2.2. MEDIA, ENZYMES AND CHEMICALS

Luria-Bertani medium (L-broth or LB): 0.5% w/v NaCl, 0.5% w/v yeast extract (Difco) and 1% w/v tryptone (Difco).

Nutrient broth (NB): 0.8% w/v Bacto nutrient medium.

TYC broth: 1% w/v tryptone, 0.5% w/v yeast extract and 0.66% CaCl_2 . Tryptone and yeast extract were autoclaved separately from CaCl_2 and 1 ml of 6.6% CaCl_2 was added to this.

2xYT broth: 0.5% w/v NaCl, 1% w/v yeast extract and 1.6% w/v tryptone.

L-agar (LA): LB containing 1.6% w/v Difco agar.

Penassay agar (PA): 2.55% w/v Difco antibiotic medium 2.

TY agar: 1.6% w/v bacto agar in TYC broth.

H-agar: 4% w/v NaCl, 5% w/v tryptone and 1.6% w/v bacto agar.

λ top-agar: 0.5% w/v NaCl, 0.5% w/v yeast extract, 1% w/v tryptone and 0.7% w/v bacto agar.

Soft H-agarose: 0.8% w/v NaCl, 1% w/v tryptone and 0.8% w/v agarose.

Minimal medium: A 1.6% w/v agar solution was autoclaved and cooled to 50°C. 10 ml of 5x minimal salts, 1 ml 20% glucose, 0.1 ml of 1M MgSO_4 and 1 ml of 10mM CaCl_2 were added. Thiamine and amino acids were added as supplements as required (0.004% w/v).

Table 2.1. List of *E. coli* strains used

Strain	Genotype/Phenotype	Source or Reference
40	$\Delta(\text{pro-lac})$ <i>trp-8 rps-2</i>	Abeles and Austin, 1987
AQ3553	F ⁻ <i>dnaA5 ilv-192 proA3 chr::pKN500</i>	Kline <i>et al.</i> , 1986
AQ3563	same as AQ3553 except <i>dnaA5</i> replaced by <i>dnaA850::Tn10</i>	Kline <i>et al.</i> , 1986
BU1416	$\Delta(\text{pro-lac})$ <i>trp-8 rps-2 dam-3</i>	Abeles and Austin, 1987
C600	F ⁻ <i>thi-1 thr-1 leuB6 lacY1 tonA21</i> <i>supE44</i> λ^-	Bachmann, 1972
C600N	same as C600 except <i>Nal^r</i>	This laboratory
C2107	<i>polA12 rha his</i>	Krevolin <i>et al.</i> , 1985
CJ225	same as CJ261 except carries pCJ100 (=F ⁻ <i>polA</i> ⁺ Cm ^r)	Joyce and Grindley, 1984
CJ261	$\Delta(\text{gal-bio})$ <i>thi-1 relA1 spoT1</i> ΔpolA Km ^r F ⁺	Joyce and Grindley, 1984
CM4722	$\Delta(\text{gal-bio})$ <i>thi-1 relA1 spoT1</i> F ⁺	Joyce and Grindley, 1984
DF1012 (RK2)	carries plasmid RK2	D. R. Helinski
DH5 α F ⁻	<i>endA1 hsdR17 supE44 thi-1</i> <i>recA1 gyrA relA1 o80 dlacZ M15</i> $\Delta(\text{lacZYA-argF})\text{U169}$	Liss, 1987
DS410	<i>minA minB rpsL</i>	Dougan and Sheratt, 1977
E177	F ⁻ <i>thr-1 leuB6 thi-1 thyA6</i> <i>deoC1 dnaA177 lacY1 strA67 tonA21</i>	Wechsler and Gross, 1971
GE1730	<i>trp::Tn10dCNS</i>	G.M. Weinstock
GM48	<i>thr leu thi lacY galK galT</i> <i>ara tonA tsx dam dcm supE44</i>	Yanisch-Perron <i>et al.</i> , 1985
GS500	same as HB101 except <i>Rif^r</i>	Selvaraj and Iyer, 1983

continued...

Table 2.1. continued

HB101	<i>F⁻ hsdS20 recA13 ara-14 proA2 lacY1 galK2 rpsL20 xyl-5 ml-1 λ⁻</i>	Bolivar <i>et al.</i> , 1977
JM105	<i>thi rpsL endA sbcB15 hspR4 Δ(lac-proAB) (F⁻ traD36 proAB lacI^qZ M15)</i>	Yansich-Perron <i>et al.</i> , 1985
LE392	<i>F⁻ hsdR514 supE44 supF58 lacY1 galK2 galT22 metB1 trpR55 λ⁻</i>	de Bruijn and Lupski, 1984
MBM7014	<i>supF</i>	G.M. Weinstock
MM383	<i>F⁻ lacZ53 rpsL151 thyA36 polA12 rha-5 deoC2 λ⁻</i>	Monk and Kinross, 1972
RR1	same as HB101 except <i>recA</i> ⁺	Bolivar <i>et al.</i> , 1977
317-1	<i>pro chr::RP4</i>	Simon <i>et al.</i> , 1983
SR1758	same as CM4722	Sharma and Smith, 1987
SR1672	same as CJ261	Sharma and Smith, 1987
VNI100	same as SR1672 but <i>trp::Tn10dCNS</i>	This study
VNI101	same as VNI100 but <i>supF</i>	This study

Table 2.2. List of non-*E. coli* strains used

Species	Source or Reference
<i>Acinetobacter calcoaceticus</i> GT16	Tardif and Grant, 1980
<i>Aeromonas hydrophila</i> AS2351	Tardif and Grant, 1980
<i>Agrobacterium tumefaciens</i> 348	Watson <i>et al.</i> , 1975
<i>Agrobacterium tumefaciens</i> A136	Watson <i>et al.</i> , 1975
<i>Alcaligenes faecalis</i> B2710	Tardif and Grant, 1980
<i>Bradyrhizobium</i> sp., IC3100	P.T.C. Nambiar
<i>Enterobacter aerogenes</i> AS1999	Tardif and Grant, 1980
<i>Oceanospirillum minutulum</i> 19193	ATCC ^a
<i>Proteus mirabilis</i> P4	This laboratory
<i>Pseudomonas cepacia</i> 25416	ATCC
<i>Pseudomonas putida</i> 2440	Schmidhauser and Helinski, 1985
<i>Pseudomonas stutzeri</i> JM300	J. Ingraham
<i>Pseudomonas stutzeri</i> PSZ-1	Krishnapillai <i>et al.</i> , 1984
<i>Rhizobium leguminosarum</i> B151	Brewin <i>et al.</i> , 1982
<i>Rhizobium leguminosarum</i> 11	Ma, Shengwu
<i>Rhizobium meliloti</i> JJ1	This laboratory
<i>Salmonella typhimurium</i> TR5530	J. Roth
<i>Salmonella enteritidis</i> AS1417	Tardif and Grant, 1980
<i>Serratia marcescens</i>	NRCC ^b
<i>Serratia liquefaciens</i> 1-141	M. Kozłowski

^aAmerican Type Culture Collection, Rockville, MD.

^bNational Research Council of Canada Collection, Ottawa, Ont.

Table 2.3. List of plasmids used^a

Plasmid	Antibiotic marker(s) ^b	Nucleotide co-ordinates of the pCU1 replicon, if any (Fig. 4.5)	Source or Reference
F ^c Cm	Cm	-	K.B. Low
pACYC184	Cm, Tc	-	Chang and Cohen, 1978
pAH69	Ap	-	Hamel, 1987
pAH322	Ap	-	"
pAT153	Ap	-	Maniatis <i>et al.</i> , 1982
pBR322	Ap, Tc	-	Bolivar <i>et al.</i> , 1977
pBR328	Ap, Cm, Tc	-	Soberon <i>et al.</i> , 1980
pBR329	Ap, Cm, Tc	-	Covarrubias and Bolivar, 1982
pCU1	Ap, Sm, Sp	1-2053	Konarska-Kozłowska and Iyer, 1981b
pCU8	Ap	1-2053	Konarska-Kozłowska and Iyer, 1981b
pCU10	Ap, Sm, Sp	1-2053	Konarska-Kozłowska <i>et al.</i> , 1983
pCU11	Ap, Sm, Sp	1-2053	Konarska-Kozłowska and Iyer, 1981b
pCU13	Ap, Sm, Sp	1-2053	"
pCU16	Ap, Sm, Sp	1-2053	"
pCU19	Ap	1-2053	Konarska-Kozłowska <i>et al.</i> , 1983
pCU28	Ap	1-2053	This laboratory
pCU31	Ap	1-2053	Konarska-Kozłowska <i>et al.</i> , 1983
pCU32	Ap	1-2053	"
pCU33	Ap, Sm, Sp	1-2053	This laboratory
pCU45	Ap, Tc	1-2053	Kozłowski <i>et al.</i> , 1987
pCU709-3	-	1-1532; 1829-2053	This study
pCU714	Km	1-2053	P. Fobert
pCU717 ^c	Km	1-2053	"
pCU718 ^c	Km	1-2053	"
pCU719 ^c	Ap, Tc	1-2053	"
pCU720 ^c	Ap, Tc	1-2053	"
pCU725	-	1-2053	This study
pCU744	Km	1-1532; 1829-2053	"
pCU751	Tc	1-1315	"

continued ...

Table 2.3. continued

pCU752	Tc	1-1593	This study
pCU773	Km	296-2053	"
pCU785	Ap, Km	1-2053	"
pCU832	Tc	1-2053	B. Love
pGS28	Ap	1-2053	Selvaraj <i>et al.</i> , 1984
pJHN11	Ap, Km, Tc	-	J.H.E. Nash
pLG338	Km, Tc	-	Stoker <i>et al.</i> , 1982
pRI91	Ap, Tc	-	This study
pRI96	Ap, Km	-	"
pRI101	Ap, Tc	1-2053	"
pRI102	Ap, Km, Tc	1-2053	"
pRI103	Ap	1-2053	"
pRI104	Ap	947-2053	"
pRI105	Ap	1375-2053	"
pRI106	Ap, Km	1-2053	"
pRI114	Ap, Tc	1-1016	"
pRI115	Cm	1-1016	"
pRI119	Ap, Tc	1017-1593	"
pRI120	Ap, Tc	1-1315	"
pRI123	Ap	1325-2053	"
pRI125	Ap, Tc	1017-1315	"
pRI128	Ap, Tc	1-1593	"
pRI129	Ap	542-2053	"
pRI133	Ap	296-2053	"
pRI145	Ap, Tc	1-1532; 1829-2053	"
pRI163	Ap	296-2053	"
pSF6	Sm, Sp	-	Selvaraj <i>et al.</i> , 1984
pUC18	Ap	-	Yanisch-Perron <i>et al.</i> , 1985
RK2	Ap, Km, Tc	-	D.R. Helinski

^aFor the deletion derivatives of pCU45 see Section 5.2.2.1

^bAp, Cm, Km, Sm, Sp and Tc indicate resistances to antibiotics ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin and tetracycline respectively.

^cThese plasmids carry base-substitution mutations within the pCU1 replicon (see Section 5.2.10).

Table 2.4. List of bacteriophages used^a

Bacteriophage	Nucleotide co-ordinates of the pCU1 replicon, if any (Fig. 4.5)	Source or Reference
$\lambda::TnphaA$	-	Manoil and Beckwith, 1985
M13mp19	-	Yanisch-Perron <i>et al.</i> , 1985
M13BOB1	1-2053	This study; Kozłowski <i>et al.</i> , 1987
M13BOB2	1-2053	"
M13BOB5	1-1532; 1829-2053	This study
M13BOB6	1-1532; 1829-2053	"
M13BOBA23	1-1315	"
M13BOBA28	1-1594	"
M13BOB2M9	542-2053	This study; Kozłowski <i>et al.</i> , 1987
M13BOB2M11	296-2053	"
M13BOB2MY3	1825-2053	"
M13BOB2MY18	947-2053	"
M13BOB2MY19	1375-2053	"
P1vir	-	G.M. Weinstock

^aOther deletion derivatives of M13BOB1 and M13BOB2 used to analyze the replicon of pCU1 are not included in this table.

Restriction enzymes were purchased from Amersham Canada Ltd, Oakville, Ont., or New England Biolabs, Beverly, MA. DNA polymerase I, Klenow enzyme, T4 DNA ligase and 1 kb MW standards of DNA (Anonymous, 1989) were purchased from GIBCO-BRL Life Technologies Ltd., Burlington, Ont. and used as recommended by the manufacturer. Exonuclease *BAL31* and the 'Cyclone' kit for obtaining deletions in double-stranded and single-stranded DNA respectively were purchased from International Biotechnologies Ltd., New Haven, CT, USA. Antibiotics and other inhibitors were purchased from Sigma Chemical Corp., USA. Isopropyl- β -D-thiogalactopyranoside (IPTG), dithiothreitol (DTT), 5-bromo-4-chloro-3-indolyl- β -D-galactoside (X-gal) and deoxyribonucleoside-triphosphates were obtained from Boehringer-Mannheim Canada Ltd.

Deoxyadenosine 5' [α - 32 P] triphosphate, triethylammonium salt, specific activity >3000 Ci/mmol, Deoxyadenosine 5'- α -[35 S] thiotriphosphate, triethylammonium salt, specific activity >1000 Ci/mmol and L-[35 S] methionine were from Amersham Canada Ltd.

2.3. BACTERIAL GROWTH CONDITIONS

Agrobacterium, *Bradyrhizobium* and *Rhizobium* species were grown in TYC broth or TY agar at 30°C. All other strains were routinely grown in LB or LA at 37°C. *E. coli* CJ261 and SR1672 were grown in NB or PA. *E. coli* DH5 α F' and JM105 were grown in 2xYT broth and JM105 was maintained on agar plates supplemented with minimal salts, glucose and thiamine. *E. coli* *polA* (*ts*) and *dnaA* (*ts*) strains were routinely grown at 30°C.

2.4. BUFFERS, SOLUTIONS AND REAGENTS

Bacterial buffer (Stoker *et al.*, 1984): 50mM Na₂HPO₄, 20mM KH₂PO₄, 70mM NaCl and 0.4mM MgSO₄.

BAL31 digestion buffer: 10mM CaCl₂, 10mM MgCl₂ and 200mM NaCl.

Poll buffer: 500mM Tris-Cl, 10mM MgCl₂ and 10mM DTT pH 7.5. Stored at -20°C.

SDS-sample buffer (Stoker *et al.*, 1984): 0.125M Tris-Cl pH 6.8, 20% v/v glycerol, 2% w/v SDS, 5% v/v 2-mercaptoethanol and 0.001% bromophenol blue.

SSC buffer, pH 6.8: 0.15M NaCl and 0.015M sodium citrate.

Sucrose buffer: 50mM Tris-Cl pH 8.3 containing 25% w/v sucrose.

TA buffer (O'Farrel *et al.*, 1980): 330mM Tris-acetate, 660mM potassium acetate, 100mM magnesium acetate, 5mM DTT and 1 mg/ml gelatine pH 8.

TAE buffer: 40mM Tris-Cl, 20mM sodium acetate and 2mM EDTA pH 7.6.

TBE buffer: 89mM Tris-Cl, 89mM boric acid and 2.5mM EDTA pH 8.2.

TE buffer: 10mM Tris-Cl and 1mM EDTA pH 8.

TES buffer: 10mM Tris-Cl, 1mM EDTA and 100mM NaCl pH 8.

Acrylamide stock solution: 38 gm acrylamide and 2 gm NN'-methylene-bis-acrylamide (bis) were dissolved in 100 ml distilled water by stirring alongwith 5 gm mixed bed resin (deionizing resin). The solution was then filtered through a Whatman No.1 filter paper and stored in a light protected container at 4°C.

DNase solution: 1 mg/ml DNase I (Sigma) was dissolved in sterile distilled water and stored at -20°C. It was thawed on ice before use.

Denaturing solution: 0.5M NaOH and 1.5M NaCl.

Denhardt's solution: 0.1% w/v Ficoll, 0.1% w/v polyvinylpyrrolidone and 0.1% w/v bovine serum albumin (Pentax fraction V, Boehringer-Mannheim). Stored at -20°C.

Hybridization solution: 6xSSC, 0.5% SDS, 5x Denhardt's solution and 100 ug/ml denatured salmon sperm DNA (Sigma). It was heated to 60°C before use.

Lysis solution: 1.25 ml 3M NaOH, 0.67 ml 10% SDS and 8.1 ml distilled water. It was prepared fresh before use.

MC solution: 10mM MgSO₄ and 5mM CaCl₂.

Neutralizing solution: 1M Tris-Cl and 3M NaCl pH 5.5.

PEG-NaCl solution: 2.5M NaCl containing 20% w/v polyethylene glycol 8000.

RNase solution: 1 mg/ml RNase A (Sigma) was dissolved in sterile distilled water and the solution heated to 90°C for 5 min to inactivate any DNases. The RNase solution was stored at -20°C and was thawed on ice before use.

Saline: 0.85% w/v NaCl solution.

Stop solution (loading dye): 0.25% w/v bromophenol blue, 100mM EDTA pH 8 and 40% w/v glycerol.

Triton X-100 lytic solution: 50mM Tris-Cl, 60mM EDTA and 2% v/v Triton X-100 pH 8.

Minimal salts (5x): 12% w/v Na₂HPO₄, 6% w/v KH₂PO₄, 1% w/v NaCl and 2% w/v NH₄Cl.

The concentrations (ug/ml) of the different antibiotics or other inhibitors used were as follows: ampicillin, 30; chloramphenicol, 30; kanamycin, 50; nalidixic acid, 50; spectinomycin, 50; streptomycin, 50 and rifampicin, 100. Higher concentrations (200 ug/ml) were used for *Pseudomonas* sp. In liquid media, the antibiotics were used at half the concentrations given above. Stocks of the antibiotics were prepared in sterile distilled water

and stored at 4°C. Nalidixic acid was dissolved in 0.2% w/v NaOH. Rifampicin was prepared fresh before use and was dissolved in methanol.

2.5. LARGE SCALE EXTRACTION OF PLASMID DNA

Isolation of large amounts of plasmid DNA was by either of the following two protocols based on the methods described by Clewell and Helinski (1969) and as modified by Humphreys *et al.* (1975).

2.5.1. Extraction of plasmid DNA from liquid cultures

The bacterial strain carrying the plasmid was grown in 500 ml of LB with appropriate antibiotic(s) to late logarithmic phase with continuous aeration. The cells were pelleted at 10,000xg for 5 min in a GSA rotor in a Sorvall RC-5B refrigerated ultracentrifuge at 4°C. The cell pellet was resuspended in 28 ml of sucrose buffer and transferred to a SS34 tube. To this cell suspension, 3 ml of 0.25M EDTA pH 8 and 1 ml of 1 mg/ml freshly prepared lysozyme solution were added and incubated on ice for 10 min. Lysis of the cells was done by the addition of 4 ml of Triton X-100 lytic solution and incubation at 37°C for about 30 min. The lysed cells were centrifuged at 35,000xg for 60 min and the DNA precipitated from the supernatant by addition of 0.3 volume of PEG-NaCl solution followed by incubation at 4°C for at least 6 h. The PEG-precipitated DNA was pelleted by centrifugation at 8000xg for 8 min. The precipitate was resuspended in 3.2 ml of TES buffer. 4 gm CsCl and 1 ml of 10 mg/ml EtBr solution were added to this solution and mixed by inversion. The refractive index of this mixture was adjusted to 1.389 with the addition of 50% w/v CsCl or TE buffer and loaded into a Beckman Quickseal

polypropylene ultracentrifuge tube. The solution was layered with mineral oil and the tube was sealed with a Beckman tube heat-sealer. The DNA was then centrifuged in a Beckman VTi80 rotor in a Beckman L8-80 ultracentrifuge at 300,000xg for 16-20 h at 15°C. At the end of the ultracentrifugation, the tube was held under longwave UV light to identify the lower band containing supercoiled plasmid DNA. This was collected from the tube using a 21 gauge needle and a syringe. EtBr was removed from this solution by successive extractions with CsCl-saturated isopropanol and the DNA dialyzed into TES buffer at 4°C. The dialyzed DNA was precipitated with 0.1 volume of 3M sodium acetate (pH 4.8) and 2 volumes of ice-cold absolute ethanol at -20°C overnight in Eppendorf tubes. This alcohol-precipitated DNA was centrifuged in a Hermle microcentrifuge at 40,000xg for 30 min, the pellet dried in a vacuum dry air oven at 50°C for 60 min and dissolved in 100-200 µl TE buffer. This DNA was stored at 4°C.

2.5.2. Extraction of plasmid DNA from cultures grown on solid media in Petri-dishes

200 µl of a fresh overnight culture of the bacterial strain in LB carrying the plasmid to be isolated was spread onto each of six LA plates supplemented with the appropriate antibiotic(s) and incubated at 37°C for 10-12 h. 3 ml of sucrose buffer was added to each plate and the cells were scraped off using a sterile glass spreader. Cell suspensions from six plates were pooled in a SS34 centrifuge tube to which 10 ml of sucrose buffer, 3 ml of 0.25M EDTA pH 8 and 1 ml of 10 mg/ml lysozyme solution were added. Cell lysis and further procedures for the isolation of plasmid DNA were performed as described above.

2.6. RAPID EXTRACTION OF PLASMID DNA

The alkaline extraction procedure of Birnboim and Doly (1979) was followed for the rapid analysis of recombinant plasmids. The bacterial strain carrying the plasmid was grown in 2 ml of LB under appropriate antibiotic selection overnight. The cells were collected by centrifugation at 8000xg in a table top centrifuge. The cell pellet was resuspended in 100 ul of TE buffer and transferred to an eppendorf microcentrifuge tube. To this, 200 ul of the lysis solution was added, mixed by inversion and incubated on ice for 5 min. At this stage, the solution became viscous and clear. 150 ul of 3M sodium acetate, pH 4.8 was then added and the mixture left on ice for 45-60 min. The gelatinous white pellet was spun down at 40,000xg for 10 min. The supernatant was collected in a fresh tube and respun at 40,000xg for 7 min. The clear supernatant was recovered and precipitated with 0.25 volume of 10M ammonium acetate and 2 volumes of ice- cold absolute ethanol at -20°C for 60 min. After centrifugation at 40,000xg for 30 min, the DNA pellet was washed in 70% v/v ethanol twice. Following the final wash, this DNA was centrifuged at 40,000xg for 15 min and the pellet dried in a vacuum dry air oven at 50°C for 60 min. The DNA pellet was dissolved in 50 ul TE buffer. 5-10 ul of this preparation was sufficient for restriction endonuclease digestion reaction and 1 ul for bacterial transformation.

2.7. LARGE SCALE EXTRACTION OF BACTERIOPHAGE M13 RF DNA

A single M13 plaque or 10 ul of the bacteriophage stock was inoculated into 5 ml of an exponentially growing culture of DH5 α F' in 2xYT broth and incubated with vigorous shaking at 37°C for 6-8 h. 4 ml of this culture was inoculated into 500 ml of 2xYT broth and incubated at 37°C for 10-12 h with aeration. The cells were collected by centrifugation

in a GSA rotor and M13 RF DNA was extracted following the same protocol described previously for the large scale extraction of plasmid DNA.

2.8. EXTRACTION OF SINGLE-STRANDED M13 PHAGE DNA (Messing, 1983)

A single M13 plaque or 10 ul of the bacteriophage stock was inoculated into 3 ml of a culture similar to the one above. This was incubated at 37°C for 8-10 h with aeration. The culture was split equally into two eppendorf tubes. The cells were pelleted in a microfuge by centrifugation at 40,000xg for 3-5 min. To 1 ml of the supernatant, 125 ul of 5M NaCl and 155 ul of 40% w/v PEG were added and thoroughly mixed by inversion. The tubes were then incubated on ice for 2 h. The phage particles were precipitated by centrifugation at 40,000xg for 10 min and resuspended in 110 ul of TE buffer. The ssDNA from the phage particles was extracted by phenol-chloroform extraction. 100 ul of buffered phenol (GIBCO-BRL) was added and the mixture vortexed gently. The aqueous and organic phases were separated by centrifugation. 100 ul of the aqueous phase was transferred to a microfuge tube containing 100 ul of chloroform:isoamylalcohol mixture (24:1) and mixed by inversion. The different phases were separated by centrifugation and the aqueous phase was transferred to a tube containing 20 ul of 10M ammonium acetate. 200 ul of ice-cold absolute ethanol was added and incubated at -20°C for 60 min. ssDNA was precipitated from this mixture and collected in 20 ul of TE buffer as described above.

2.9. RECOVERY OF DNA FROM AGAROSE GELS

For the extraction of DNA from agarose gels, the gels were prepared and electrophoresed in TAE buffer. After EtBr staining of the gel in distilled water, the gel was

illuminated under longwave UV light. The gel fragment containing the DNA band of interest was sliced out of the gel using a razor blade and transferred to a clean microfuge tube. DNA was recovered from this piece of agarose gel using the 'GeneClean' kit purchased from Bio101 Inc., La Jolla, CA following the protocol recommended by the manufacturer. Briefly, the agarose was dissolved in NaI buffer and the DNA precipitated with "Glassmilk". The precipitate was then washed thrice with NEW (NaCl, ethanol and water) buffer and DNA eluted in TE buffer.

2.10. *IN VITRO* MANIPULATION OF DNA

2.10.1. Restriction enzyme digestion and ligation

Restriction enzyme digestions of DNA were performed in TA buffer at 37°C for 2 h. Heat inactivation of the digested products was done at 65°C for 5 min and the samples were immediately transferred on ice. Digestion with endonuclease *Sma*I was done in a buffer with the following composition: 20mM KCl, 10mM Tris-Cl pH 8, 10mM MgCl₂ and 1mM DTT (Maniatis *et al.* 1982). 0.1 volume of the stop dye was added to the restricted products before loading on an agarose gel for electrophoresis. 1 kb DNA ladder (GIBCO-PRL) and *Hind*III digested λ DNA were used as MW standards.

Ligation of restricted DNAs to construct recombinant DNA molecules was done using T4 DNA ligase. The 5x ligase buffer supplied by GIBCO-BRL was used in such reactions. Ligations were done at 15°C for 16-20 h. The ligation mixture was stored at --20°C until use.

2.10.2. Construction of deletions in ds- and ssDNA

Deletions in plasmid DNA were generated using exonuclease *BAL31*. The experimental conditions were as recommended by IBI. Plasmid DNA (about 3-5 ug) was at first linearized by cleavage with a restriction enzyme and ethanol-precipitated as described before. The DNA was then resuspended in *BAL31* digestion buffer, to which 1-3 U of the enzyme was added and incubated at 37°C for varying periods of time. The extent of the deletions over a progressive period of time was monitored by drawing aliquots of the reaction mixture at different time intervals and analyzing them by agarose gel electrophoresis. The deleted DNA fragments in the samples were then "end-filled" using all four dNTPs in a Klenow fragment-catalyzed reaction and ligated as described above. The samples were then used in a bacterial transformation experiment for further characterization.

Deletions in ssDNA were obtained following the procedure of Dale *et al.* (1985) using the 'Cyclone' kit purchased from IBI. An oligonucleotide primer (RD 22mer, see Table 2.5) was annealed to the single-stranded template and digested with *EcoRI* at 42°C. A nested series of deletions was obtained by exposing this DNA to T4 DNA polymerase in the absence of all four dNTPs by incubation of the reaction mixture at 37°C for varying lengths of time. The DNA was then dC-tailed with terminal nucleotidyl transferase, annealed with the 22mer and ligated as described above. This mixture was used in a transformation experiment of *DH5 α F'* and the extent of the deletions were ascertained at first by comparison of the size of the phage DNAs isolated from the plaques obtained in the transfection experiment to the wild type DNA and subsequently by DNA sequencing.

Table 2.5. List of oligonucleotide primers used

Designation	DNA sequence	Source or Reference
P1	5'-CAATGAGTTATATTAGCTCT	Kozlowski <i>et al.</i> , 1987
P2	5'-TTCGCTTACTGAAGGCTGTG	"
P3	5'-AATTGAGCAAGACTTTGCAT	"
P4	5'-AATTCGCCCATCTTCATGAC	"
P5	5'-AGGTATAATTAAAGTTATGG	"
K2	5'-GCGCCGTACTGCGAGTTAG	This study
MK 20mer	5'-ACCAGGCTATCAAGGATGTT	V. Thatte
RD 22mer	5'-CGACGGCCAGTGAATTCCCCCC	IBI, Inc.
Universal primer	5'-GTAAAACGACGGCCAGT	USB Corpn.
'-40' primer	5'-GTTTTCCCAGTCACGAC	"

2.10.3. Labeling of 'probe' DNA by nick translation

1 ug of the plasmid DNA to be labeled was mixed with 1.5 ul of 5mM solutions of dCTP, dGTP and dTTP respectively in a sterile microfuge tube. 5 ul of 50uM dATP and 5uCi of ^{32}P labelled dATP were then added. This was brought to 26 ul with the addition of distilled water followed by the addition of 1 ul of DNase solution (1 ug/ml) and 5U of DNA polymerase I enzyme. This reaction mixture was incubated at 15°C for 2 h. The reaction was stopped by adding 5 ul of 0.25M EDTA pH 8. The unincorporated nucleotides were removed by ammonium acetate-ethanol precipitation. This was done by the addition of 17 ul of 10M ammonium acetate and 2 volumes of ice-cold absolute ethanol followed by incubation at -20°C. DNA was pelleted and eluted in TE buffer as described previously. Immediately before hybridization, this labeled 'probe' was denatured by placing the tube in a boiling water bath for 5 min. The tube was then removed and placed quickly on ice for 10 min and added directly to the hybridization bag.

2.10.4. DNA sequence analysis

DNA sequencing was done using the 'Sequenase' kit purchased from United States Biochemical Corporation (USB), Cleveland, OH and according to the manufacturer's suggested protocol. The principle of the procedure is based on the di-deoxy chain termination method described by Sanger *et al.* (1977). An appropriate primer (Table 2.5) was annealed to the template by heating the primer-template mixture to 65°C and slow-cooling to 30°C. The labeling reaction with ^{35}S labeled dATP and other non-radioactive dNTPs was done at 25°C for 3-5 min and the sequencing reaction with a mixture of the respective dNTPs and ddNTPs using 'Sequenase' Version 2.0 at 37°C for 5-8 min. The reactions were stopped by the addition of the stop solution containing formamide provided

with the kit and denatured in boiling water before being fractionated on a polyacrylamide gel. The samples prepared for the sequencing reaction were either used immediately or stored at -20°C overnight before use.

2.11. C-TEST (Messing, 1983)

This test was done to identify recombinant M13mp19 bacteriophages that carried complementary strands of a given DNA fragment. Only one type of the strand of M13 phages are extruded in the viral particles. Hence, any hybridization between two recombinant M13 phages must be *via* the cloned inserts of DNA which by definition must be in complementary orientations. To detect any such hybridization, 20 μ l of each of the recombinant phages were first mixed with 2 μ l of 10% w/v SDS and 2 μ l of the stop solution and heated to 65°C for 10 min. The mixture was then slow-cooled to 30°C. The samples were then electrophoresed on an agarose gel. Both the test phages prepared in the same way without inclusion of the second phage were also electrophoresed as controls. If the recombinant bacteriophages contained complementary DNA sequences in the cloned segments, they hybridized and migrated slower than the bacteriophage DNA alone in the agarose gel.

2.12. 'SOUTHERN' HYBRIDIZATION (Southern, 1975)

The target species of DNA was at first restricted with appropriate restriction enzymes and separated on an agarose gel by electrophoresis. The gel was stained with EtBr and photographed as outlined above. The nucleic acids in the gel were then blot-transferred to a Hybond-N nylon membrane (Amersham Canada Ltd.) by capillary transfer (Southern,

1975; Maniatis *et al.*, 1982) as follows. The agarose gel was exposed to long wave UV light for 10 min to allow depurination. This facilitates efficient transfer. The gel was soaked in denaturing solution for 30 min and then in the neutralizing solution for 30 min with gentle agitation. The gel was then placed on a glass plate over three 3MM Whatman papers, the ends of which were immersed in 20xSSC buffer. The gel was then covered with a strip of Hybond-N membrane over which five strips of 3MM Whatman papers cut to the size of the gel and a stack of paper towels were placed. The capillary transfer was allowed to proceed overnight. The membrane was then removed, air-dried and exposed to longwave UV light for 10 min to 'fix' the DNA on the membrane.

The membrane with the DNA was prehybridized in 10 ml of hybridization solution at 65°C for 2 h in a sealed plastic bag. The nick-translated probe was added to this and hybridization was done at 65°C overnight. The hybridized membrane was washed twice in 2xSSC at 60°C for 30 min each, once in 0.1xSSC and 0.1% w/v SDS at 60°C for 15 min and once in 0.1xSSC at 60°C for 15 min. The membrane was then air-dried and autoradiographed as described in Section 2.15.

2.13. ANALYSIS OF THE PLASMID DNA ENCODED POLYPEPTIDE PRODUCTS

2.13.1. Preparation of 'mini-cells' and labeling of proteins (Stoker *et al.*, 1984)

The plasmid whose protein product(s) were to be analyzed was at first transformed into the 'mini-cell' producing strain *E. coli* DS410. The plasmid-bearing strain was then grown in LB overnight under appropriate selective condition(s). The cells were then pelleted in a GSA rotor at 5000xg for 5 min at 4°C. The supernatant was collected in a

fresh tube and spun again at 12,000xg for 15 min. The cell pellet was resuspended in 6 ml of minimal medium and layered on a 10-30% w/v sucrose gradient. The gradient was centrifuged in a HB4 rotor at 8000xg for 20 min at 4°C. The top two-thirds of the 'mini-cell' band was collected, concentrated by centrifugation and repurified on a 10-30% w/v sucrose gradient twice as described above. Finally, the 'mini-cells' were concentrated and stored at -70°C in minimal medium containing 30% v/v glycerol.

In order to label the plasmid encoded protein(s), the following procedure was used. The 'mini-cells' were resuspended in 200 ul of minimal medium and 3.5 ul of 10.5% w/v Difco methionine assay medium was added. This mixture was incubated at 37°C for 90 min. 25 uCi of ³⁵S labeled methionine was added to this and the incubation continued for another 60 min. 10 ul of non-radioactive L-methionine (8 mg/ml) was then added and incubated for a further period of 5 min. The cell suspension was then centrifuged at 40,000xg for 5 min and the supernatant discarded. The pellet was resuspended in 50 ul of bacterial buffer. 50 ul of SDS-sample buffer was added to this and heated in a boiling water bath for 5 min. 25 ul of this mixture was used for analysis in an SDS-polyacrylamide gel. Pre-stained proteins (Bio-rad) or ¹⁴C-labelled proteins (GIBCO-BRL) were used as MW standards to calculate the MW of the proteins produced by the plasmids.

2.13.2. *In vitro* transcription and translation assay

Coupled transcription and translation of plasmid DNA was performed *in vitro* using the 'Prokaryotic DNA mediated translation' kit purchased from Amersham Canada Ltd. and according to the procedure of De Vries and Zubay (1967) and as described by Zubay (1973). 5 ug of plasmid DNA in 5 ul of TE buffer was mixed with 5 ul of the S-30 extract prepared from *E. coli* strain MRE600 and 7.5 ul of the 'Supplement' solution. The S-30

fold into non-selective medium and grown until the culture reached visible turbidity (approximately 10^7 cfu/ml). This corresponded to about 15 cell generations. Successive dilutions and growth were continued in experiments when a higher number of cell generations were studied. Following this, the bacterial culture was diluted in saline and plated on non-selective agar to obtain several single colonies. At least 100 of these colonies were picked on non-selective and appropriate selective agar plates. The fraction of colonies expressing antibiotic resistance were thus determined. Expression of the respective antibiotic resistance was inferred as being indicative of the continued presence of the plasmid in the strain. In some experiments where indicated in Chapters 3-5 the physical presence of the plasmid was demonstrated.

2.20. PLASMID INCOMPATIBILITY TEST

These experiments were done in the *E. coli recA* strains HB101 or GS500. The target plasmid, the incompatibility status of which was being examined was at first introduced into HB101 by transformation or conjugation. The test plasmid, against which the expression of incompatibility by the target plasmid was being tested was subsequently introduced into this strain. At this stage, selection was imposed for the incoming plasmid. At least 60 transformants or transconjugants were then picked on non-selective media and grown. Presence of the 'incoming' or the 'resident' plasmids in these colonies was tested by replica plating colonies onto selective plates for the two plasmids respectively. The expression of the appropriate antibiotic- resistance was inferred as being indicative of the strain carrying the plasmid. Colonies from these plates were then replica-plated onto plates containing the antibiotic of the 'second' plasmid. Expression of the 'second' plasmid's phenotype was concluded as non-expression of incompatibility between the two plasmids in

extract and the 'Supplement' solution provide the requisite enzymes, appropriate nucleotides, tRNA, energy sources and inorganic salts for the transcription and translation reactions. To the above mixture, 2 ul of ^{35}S methionine and 3 ul of other unlabeled amino acids were added and incubated at 37°C for 60 min. 5 ul of methionine chase solution was then added and the incubation continued for a further 5 min at 37°C . The reactions were then stopped by the addition of 20 ul of the SDS-sample buffer. 5 ul of this end product was used in a SDS-polyacrylamide gel for separation of the polypeptides (Section 2.14.2).

2.14. GEL ELECTROPHORESIS

2.14.1. Agarose gel electrophoresis

The Bio-rad mini wide-sub Cell electrophoresis unit was routinely used. Small gels (5x7.5 cm) and large gels (10x15 cm) were used to separate DNA fragments in 0.7% or 1.2% w/v agarose dissolved in TBE. The small gels were electrophoresed at 100V for about 60 min and the large gels at 70V for about 3 h. The current for electrophoresis was supplied by a regulated power supply power pack from Savant Instruments Inc. Model C^{VR} 300. After electrophoresis, gels were stained in an aqueous solution of EtBr (1 ug/ml) for 30 min and destained in distilled water for 30-60 min. Gels were illuminated over longwave (approximately 360nm) UV light and photographs taken with a polaroid MP4 vertical land camera using a red filter and polaroid type 57 film.

2.14.2. Polyacrylamide gel electrophoresis

The BRL sequencing gel unit MS2 and polyacrylamide gels were used to fractionate DNA. Vertical 7.5% w/v polyacrylamide gels were composed of 15 ml of acrylamide stock solution, 10 ml of 10xTBE buffer, 42 g ultrapure urea, 1 ml of 10% w/v ammonium

persulfate and 60 μ l of Temed (N,N,N',N'-tetramethylethylenediamine). Alternatively, 8% polyacrylamide gels were prepared from 'Gelmix' (GIBCO-BRL) following addition of 450 μ l of 10% w/v ammonium persulfate to facilitate polymerization as directed by the supplier. Gels were cast between 30x34 cm glass plates using 0.3 mm Mylar side-spacers and were allowed to polymerize at least 2 h before use. Mylar "sharks-teeth" combs were used to form wells at the top of the gels. The gels were prerun for at least 60 min at 900V. Electrical current was supplied by an LKB 2297 macrodrive 5 constant power pack. The samples were then loaded and the gels run at 1200-1500V as required. The gels were 'fixed' in 10% v/v acetic acid and 10% v/v methanol for 30 min and dried in a slab gel drier at 80°C for 60 min.

For protein gels, the discontinuous electrophoresis system of Laemmli (1970) was followed. The Bio-rad Protean II gel apparatus was used for this purpose. Proteins were separated in a resolving gel containing 15% w/v acrylamide, 0.1M sodium phosphate pH 7.2, 0.1% w/v SDS and 6M ultrapure urea. A 3.5% w/v stacking upper gel was formed on top of the resolving gel using the same buffer conditions. The electrophoresis buffer was 0.1M sodium phosphate pH 7.3 and 0.1% w/v SDS (Anonymous, 1984). The gels were run at 60V until the tracking dye reached three-fourths distance to the bottom of the gel. The gels were fixed in 45% v/v ethanol, 10% v/v glacial acetic acid and 1% v/v glycerol for 30 min and then immersed in a solution of 'Enlightning' (NEN Research Products, DE, USA) before being dried in a slab gel drier at 60°C for at least 2 h.

2.15. AUTORADIOGRAPHY

The dried DNA sequencing polyacrylamide gel on 3MM Whatman paper was exposed to Kodak X-OMAT AR diagnostic film in a 35x43 cm X-ray cassette at room

temperature overnight. Protein gels were exposed to Hyperfilm β -max (Amersham) using DuPont intensifying screens at -70°C for at least 24 h. 'Southern' hybridization filters were exposed to Kodak X-OMAT AR films using DuPont intensifying screens at -70°C for at least 60 min. The X-ray films were developed using an AFP Imaging 14XL X-ray film processor. Developer and Fixer for this purpose were purchased from Picker Chemicals and used as recommended by the manufacturer.

2.16. TRANSFORMATION AND TRANSFECTION OF *E.coli*

'Competent' cells of *E.coli* were made according to the procedure of Cohen *et al.* (1972) with slight modifications. An overnight culture of the appropriate *E.coli* strain grown in selective LB was diluted 100-fold and cultivated in LB containing 10mM MgCl_2 to an O.D. of 75 (as calibrated in a Klett-Summerson photoelectric colorimeter with a blue filter No. 42). For most strains this corresponded approximately to 2×10^7 cfu/ml. The cells were collected by centrifugation at $8000\times g$ for 7 min at 4°C in a bench top centrifuge and washed twice in 75mM CaCl_2 . After the final wash, the cells were resuspended in 0.2 volume of 75mM CaCl_2 and 100 μl of this was used for transformation with 100-200 ng of plasmid DNA or for transfection with 10-50 ng of bacteriophage DNA. The cells were incubated with the DNA on ice for 60 min, heat-pulsed at 37°C for 5 min, chilled on ice for 5 min and diluted 10-fold with LB. This was incubated at 37°C for 60 min before plating on selective media. When selecting for ampicillin-resistant (Ap^r) transformants, this 'expression' period was reduced to 45 min before plating. All procedures of incubation and heat-pulse were done at 30°C in experiments involving *E. coli* C2107. For bacterial transfection, 100 μl of the cells in the logarithmic phase of the same strain as used in the transfection experiment, 20 μl of 24 mg/ml IPTG dissolved in sterile distilled water and 10 μl of 100

mg/ml X-gal dissolved in dimethyl-formamide were added to the transfection mixture after the heat-pulse and chilling steps and mixed with 3 ml of soft H-agarose and was overlaid on H-agar. The recombinant bacteriophages were identified by their ability to form colorless plaques on X-gal medium while the wild type phages produced blue plaques.

2.17. TRANSDUCTION OF *E.coli*

2.17.1. P1 transduction

An overnight culture of the donor strain was diluted 50-fold and grown in a 5 ml volume of LB at 37°C for 60 min. 0.1 ml of 0.25M CaCl₂ was added to this and incubated at room temperature for 5 min. Then P1_{vir} was added to this at an approximate moi (multiplicity of infection) of 0.1. The cells were incubated at 37°C until lysis was complete (about 2 h). The phage particles were collected by the addition of 0.25 ml of chloroform and removal of the cell debris by centrifugation at 8000xg for 10 min in a bench top centrifuge.

The recipient cells were diluted 50-fold in 5 ml of LB and grown at 37°C for 2 h. The cells were then collected by centrifugation as described above and resuspended in 5 ml of MC salts solution. 0.2 ml of the recipient culture and 0.1 ml of the P1 phage stock prepared on the donor culture were mixed and incubated at 37°C for 20 min. An equal volume of 2M sodium citrate was added and incubated at room temperature for 5 min. The volume was brought to 5 ml with LB or NB and incubated with aeration at 37°C for 2 h. The cells were spun down at 8000xg, resuspended in 0.05 volume and plated on selective media.

2. λ transduction

Generally, propagation of λ phage particles was done in *E.coli* LE392. λ particles were added at an moi of 0.1 to 0.2 ml of stationary phase recipient cells grown in 0.2% w/v maltose. This was incubated at 30°C until visible cell turbidity dropped (approximately 40 min). 5 ml of LB and 0.2% w/v maltose were then added and incubation continued with aeration at 30°C overnight. The cells were removed by centrifugation and the supernatant containing the phage particles was filter-sterilized through a 0.45 μ M Millipore filter unit and stored at 4°C. The phage titer was estimated by mixing dilutions of the stock preparation with 1 ml of the *E.coli* LE392 cells in exponential phase in 2 ml of top-agar and casting this as an overlay on LA plates. The plates were incubated at 37°C overnight and the plaques counted and expressed as pfu/ml.

2.18. BACTERIAL CONJUGATION

2×10^8 cells of the donor and 2×10^7 cells of the recipient at exponential phase were mixed and concentrated to 0.1 volume (0.1 ml). This was spotted on a 0.45 μ M Millipore filter disc on an LA plate and incubated at 30°C for 2 h. Bacterial matings involving *Agrobacterium*, *Bradyrhizobium* and *Rhizobium* sp. were extended to 16-20 h. The filter discs were then immersed in 1 ml of 0.85% w/v saline, agitated by vigorous vortexing and dilutions spread on selective media.

2.19. PLASMID STABILITY TEST

The bacterial strain harboring the plasmid was inoculated into the appropriate culture medium under appropriate selective conditions overnight. This was then diluted 100-

fold into non-selective medium and grown until the culture reached visible turbidity (approximately 10^7 cfu/ml). This corresponded to about 15 cell generations. Successive dilutions and growth were continued in experiments when a higher number of cell generations were studied. Following this, the bacterial culture was diluted in saline and plated on non-selective agar to obtain several single colonies. At least 100 of these colonies were picked on non-selective and appropriate selective agar plates. The fraction of colonies expressing antibiotic resistance were thus determined. Expression of the respective antibiotic resistance was inferred as being indicative of the continued presence of the plasmid in the strain. In some experiments where indicated in Chapters 3-5 the physical presence of the plasmid was demonstrated.

2.20. PLASMID INCOMPATIBILITY TEST

These experiments were done in the *E. coli recA* strains HB101 or GS500. The target plasmid, the incompatibility status of which was being examined was at first introduced into HB101 by transformation or conjugation. The test plasmid, against which the expression of incompatibility by the target plasmid was being tested was subsequently introduced into this strain. At this stage, selection was imposed for the incoming plasmid. At least 60 transformants or transconjugants were then picked on non-selective media and grown. Presence of the 'incoming' or the 'resident' plasmids in these colonies was tested by replica plating colonies onto selective plates for the two plasmids respectively. The expression of the appropriate antibiotic- resistance was inferred as being indicative of the strain carrying the plasmid. Colonies from these plates were then replica-plated onto plates containing the antibiotic of the 'second' plasmid. Expression of the 'second' plasmid's phenotype was concluded as non-expression of incompatibility between the two plasmids in

question. Incompatible plasmids triggered loss of one plasmid or the other in the same cell in the absence of selection for maintenance of both. The whole experiment was repeated again with one modification. The order of entry of the two plasmids into the *E.coli* was reversed i.e., the 'resident' plasmid of above was used as the 'incoming' plasmid and the 'incoming' plasmid as the 'resident' plasmid.

2.21. TEST FOR THE DEGREE OF PLASMID-ENCODED ANTIBIOTIC-RESISTANCE

E. coli HB101 carrying the appropriate plasmid was grown overnight in LB with selection. Using the flat end of a sterile toothpick, the culture was streaked on LA containing different concentrations of the test antibiotic. The LA plates were incubated at 37°C for 24 h and bacterial growth was examined. The concentration at which no bacterial growth was observed was taken to be the minimum concentration of the antibiotic inhibitory to the growth of the plasmid-carrying strain.

2.22. TEST FOR UV-LIGHT SENSITIVITY OF BACTERIA

The bacterial culture was grown to a concentration of about 10^8 cfu/ml in nutrient broth. 10 ml aliquots were harvested in sterile SS34 tubes by centrifugation at 8000xg for 5 min at 4°C. The cells were resuspended in 10 ml of 0.85% w/v saline. 2 ml of the cell suspension was then placed in a 10 cm diameter by 1.5 cm high sterile glass Petri dish and exposed to a UV light source (with the cover of the Petri dish removed) for an appropriate dose. 0.5 ml of the UV-exposed cell suspension was withdrawn and dilutions spread on PA. These PA plates were incubated in the dark at 37°C for 36 h and per cent survivors was

estimated. The source of the UV light was a 50W germicidal lamp. The dose rate of the UV light delivered was 10 ergs/sq.mm/sec and was monitored using an IL600A Research Photometer and an I6620 photodensitometer (International Light Inc., Newburyport, MA, USA).

2.23. COMPUTER ASSISTED ANALYSIS OF DNA SEQUENCES

Computer assisted analysis of the DNA sequences for the location of restriction sites, ORFs and consensus nucleotide stretches was done using the Zenith data systems IBM PC-XT compatible hardware and the 'Microgenie' software program developed and sold by Beckman Instruments Inc., Palo Alto, CA, USA.

CHAPTER 3

Host-Ranges of pCU1 and its Replicon

in Gram-negative purple bacteria

3.1. INTRODUCTION

The most efficient means of lateral spread of plasmids in bacterial populations is by conjugation. Quite expectedly then, plasmids that provide their own functions for conjugative transfer can potentially spread to a vast variety of bacterial species. The genetic regions responsible for the transfer functions can be broadly divided as the *cis*-acting *oriT*, that serves as the recognition and nicking site for transfer of plasmid DNA during conjugation and the *trans*-acting pilus-synthesizing and DNA-processing regions (for a review see Willetts and Wilkins, 1984). The *oriT* has also been called *bom* (*basis of mobility*), e.g., in plasmid ColE1 (Warren *et al.*, 1979). Plasmids like ColE1 and RSF1010 do not provide all functions required for self-transfer (Willetts and Wilkins, 1984). They are 'mobilized' when the transfer functions of certain other 'helper' plasmids (e.g., F and RK2 respectively) are provided *in trans*. Thus, the ability of such plasmids not carrying all *tra* functions to spread laterally will be more limited and is absolutely dependent upon conditions that provide the cognate transfer functions. Plasmids that lack even the *oriT* spread poorly in a bacterial population by the natural modes of gene transfer. Interbacterial transfer of plasmids is also possible during generalized transduction by bacteriophages such as P1. Small plasmids may not be transported efficiently by this mode because the bacteriophage 'head' must be filled with a minimum amount of DNA before packaging and extrusion (e.g. in bacteriophage λ , Feiss *et al.*, 1977). In several instances, the size of the plasmid DNA is far too small to be accommodated in a phage head. Since plasmids, in nature, are always carried within a bacterial cell, natural 'transformation' is a less likely mode for the horizontal propagation of plasmids. In the laboratory however, artificial ways of making 'competent' bacteria

(Cohen *et al.*, 1972; Hanahan, 1987) have enabled introduction of foreign plasmids into different bacteria almost at will.

Whatever the means of plasmid entry into a 'recipient' cell, stable inheritance of the plasmid in the recipient is critical to the survival of the plasmid as a separate entity. This requires the ability of the plasmid to replicate in the new environment (the recipient) and to ensure that every daughter cell receives at least one copy of the plasmid molecule following cell division. This requires an ability of the plasmid to recognize and utilize the genetic functions encoded by the recipient bacterial species: second level at which the spread of a plasmid could be checked. Bacteria into which a plasmid can be transmitted and in which the plasmid is stably maintained without any apparent selection for it constitute the plasmid's host-range.

IncN group of plasmids are conjugative and have been isolated from different Gram-negative bacteria like *Escherichia*, *Salmonella*, *Shigella*, *Proteus* and *Providencia* sp. (Shapiro, 1977; Hedges *et al.*, 1973). In the laboratory, they can be transferred to *Caulobacter* (Ely, 1979) and *Myxococcus* sp. (Parish, 1975). A clear understanding of the host-range of IncN plasmids is necessary to understand the basis of such a broad host-range and to be able to use them as cloning vectors. Here, a study on the host-range of a representative IncN group plasmid, pCU1 (Konarska-Kozłowska and Iyer, 1981a) and of its mini-derivative, pCU785, containing its replicon is presented.

3.2. RESULTS

3.2.1. Construction of pCU785

In order to examine the host-range of the replicon of pCU1 and to compare it to the host-range of pCU1, an efficient delivery system of the replicon of pCU1 into the test bacteria is essential. The IncP plasmid RK2 has been shown to exhibit a broad host-range (Schmidhauser, 1986) and its transfer functions are promiscuous (Guiney and Lanka, 1989). The genetic functions of the RK2 *tra* system contain the *cis*-acting, *oriT* and the *trans*-acting DNA segments (Guiney and Yakobson, 1983; Guiney and Lanka, 1989). Therefore, a mini-derivative of pCU1 containing its replicon, *oriT* of RK2 and suitable selectable markers was constructed (=pCU785). The presence of *oriT* of RK2 in pCU785 enables its conjugative mobilization when the RK2 *tra* functions are provided *in trans* (see Sections 3.2.2. and 3.2.3).

Plasmid pCU45 (Kozlowski *et al.*, 1987) contains the minimal replicon of pCU1 cloned into pBR329. The smaller *EcoRI-PstI* fragment of pCU45 was replaced with the smaller *EcoRI-PstI* fragment of pSF6 (Selvaraj *et al.*, 1984) containing the *oriT* of RK2 to yield pRI101. The *HindIII* internal fragment of Tn5 containing the *Km^r* gene in pJHN11 was then cloned into the unique *HindIII* site of pRI101 to obtain pRI106. pCU785 was then constructed by cloning the 2 kb *BamHI* fragment of pRI106 into the single *BamHI* site of pGS28 (Selvaraj *et al.*, 1984). pGS28 is a clone of a deletion derivative of pCU1, pCU32 (Kozlowski *et al.*, 1987) and *oriT* of RK2. The construction of pCU785 is also depicted in Fig. 3.1.

Fig. 3.1. Construction of the mobilizable IncN mini-derivative pCU785. Sizes of the plasmids (in kb) are: pSF6, 11.1; pCU45, 6.2; pRI101, 6.2; pJHN11, 10.1; pRI106, 9.5; pGS28, 7.8; pCU785, 9.9. Hatched areas and shaded areas represent the pCU1-*rep* and RK2-*oriT* regions respectively. Abbreviations: Ap^r, Km^r, Sm^r, Sp^r and Tc^r, ampicillin, kanamycin, streptomycin, spectinomycin and tetracycline resistance markers respectively; B, E, H and P, restriction sites for *Bam*HI, *Eco*RI, *Hind*III and *Pst*I respectively.

3.2.2. Transfer of pCU785 between *E. coli* strains

E. coli S17-1(pCU785) was used as the donor strain of the plasmid pCU785. S17-1 contains plasmid RP4 (similar or identical to RK2, Burkhardt *et al.*, 1979) integrated in the bacterial chromosome (Simon *et al.*, 1983). The frequency of mobilization of pCU785 to recipient *E. coli* GS500 is shown in Table 3.1. Frequencies of mobilization of pRI91, pRI96, pRI101 and pRI106 from S17-1 carrying them respectively to GS500 is also included in Table 3.1. Constructions of pRI91 and pRI96 are shown in Figs. 3.2 and 3.3 respectively. pRI91 was used as a control for the pBR replicon, *oriT* of RK2 and the antibiotic-marker of pRI101 and pRI96 for the same genetic functions of pRI106. In other words, the essential difference between (a) pRI91 and pRI101 and (b) pRI96 and pRI106 is the additional presence of the pCU1 minimal replicon in pRI101 and pRI106. It should be noted that no transfer ($<10^{-9}$ per recipient) of the RP4-determined markers was observed in matings between S17-1 and GS500.

3.2.3. Transfer of pCU1, pCU785 and RK2 to other Gram-negative bacteria

E. coli C600(pCU1), *E. coli* S17-1(pCU785) and *E. coli* DF1012(RK2) were used as the donors in bacterial mating experiments with the bacteria listed in Table 2.2. The range of transfer frequencies obtained is shown in Table 3.2. Transcipients are defined as the recipients that express the respective plasmid-encoded antibiotic marker following conjugation. Expression of a second antibiotic marker encoded by the plasmid was done whenever possible.

pRI91, pRI96, pRI101 and pRI106 were mobilized to *A. tumefaciens* 348 and *R. meliloti* at a frequency of 10^{-2} - 10^{-3} .

Table 3.1. Frequency of mobilization of pCU785, pRI91, pRI96, pRI101 and pRI106 between *E. coli* strains

Donor	Frequency of mobilization ^a to <i>E. coli</i> GS500
S17-1(pCU785)	0.06
S17-1(pRI91)	0.04
S17-1(pRI96)	0.04
S17-1(pRI101)	0.04
S17-1(pRI106)	0.04

^aTranscript frequencies were calculated as described in the footnote to Table 3.2.

Fig. 3.2. Construction of pRI91. Sizes of the plasmids (in kb) are: pAH69, 4.1; pAH322, 4.3; pRI91, 5.5. *oriT* and *cos* represent the RK2 *oriT* regions and bacteriophage λ *cos* region respectively. Abbreviations: Ap^r and Tc^r, ampicillin and tetracycline resistance markers respectively; E, restriction site or restriction enzyme digestion with *EcoRI*.

Fig. 3.3. Construction of pRI96. Sizes of the plasmids (in kb) are: pAH69, 4.1; pJHN11, 10.1; pRI96, 7.4. Symbols and abbreviations as described in Fig. 3.2 except that Km^r indicates kanamycin resistance marker and H indicates restriction site or restriction enzyme digestion with *Hind*III.

TABLE 3.2. Range of transcript frequencies for pCU1, pCU785, and RK2^a

Species	Range of transcript frequency ^b for:		
	pCU1	pCU785	RK2
Alpha subdivision			
<i>Agrobacterium tumefaciens</i> 348 ^c	1×10^{-3} to 4×10^{-3}	3×10^{-3} to 5.5×10^{-3}	9.5×10^{-3} to 6×10^{-4}
<i>Agrobacterium tumefaciens</i> A136 ^c	7.5×10^{-8} to 9×10^{-8}	NT ^d	NT
<i>Bradyrhizobium</i> sp. ^c	5×10^{-7} to 6×10^{-7}	5×10^{-3} to 7.5×10^{-3}	NT
<i>Rhizobium leguminosarum</i> B151	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	NT
<i>Rhizobium leguminosarum</i> 11	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	NT
<i>Rhizobium meliloti</i>	1.5×10^{-7} to 2.5×10^{-7}	2×10^{-4} to 3×10^{-4}	4×10^{-4} to 6×10^{-4}
Beta subdivision			
<i>Alcaligenes faecalis</i>	$<1 \times 10^{-9}$	3.5×10^{-7} to 4×10^{-7}	8×10^{-4} to 9×10^{-4}
<i>Pseudomonas cepacia</i>	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	1.5×10^{-3} to 3×10^{-3}
Gamma subdivision			
<i>Acinetobacter calcoaceticus</i>	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	4×10^{-8} to 8×10^{-7}
<i>Aeromonas hydrophila</i>	4×10^{-6} to 3.5×10^{-4}	3×10^{-6} to 9×10^{-5}	NT
<i>Enterobacter aerogenes</i>	2×10^{-3} to 7×10^{-2}	4×10^{-4} to 8.5×10^{-3}	NT
<i>Oceanospirillum minutulum</i>	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
<i>Proteus mirabilis</i>	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	1×10^{-6} to 4×10^{-4}
<i>Pseudomonas putida</i>	$<1 \times 10^{-9}$	1×10^{-8} to 2.5×10^{-8}	2×10^{-3} to 8×10^{-4}
<i>Pseudomonas stutzeri</i> JM300	0.5×10^{-8} to 3×10^{-6}	$<1 \times 10^{-9}$	4×10^{-6} to 4×10^{-6}
<i>Pseudomonas stutzeri</i> PSZ-1	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$	6×10^{-7} to 4×10^{-6}
<i>Salmonella typhimurium</i>	2.5×10^{-6} to 9×10^{-4}	1.5×10^{-6} to 7×10^{-6}	NT
<i>Salmonella enteritidis</i>	1×10^{-6} to 3×10^{-4}	1.5×10^{-4} to 4×10^{-4}	5×10^{-3} to 9×10^{-3}
<i>Serratia marcescens</i>	7×10^{-6} to 9×10^{-6}	NT	NT
<i>Serratia liquefaciens</i>	1×10^{-4} to 5×10^{-4}	1×10^{-4} to 6.5×10^{-4}	NT

^a For definition of transcript, see Section 3.2.3.^b Transcript frequency is the number of transcripts obtained per recipient. $<1 \times 10^{-9}$ indicates that no transcripts were obtained.^c NaF strains were used in these mating experiments. In all other cases Rif strains were used.^d NT, Not tested.

3.2.4. Maintenance of pCU1 and pCU785 in the transciplants

Stable inheritance of pCU1 and pCU785 in the transciplants was examined as described in Section 2.19. Table 3.3 provides the results of the stability tests.

While pRI91 and pRI96 were lost from both *Agrobacterium* and *Rhizobium* sp. even under selective conditions, pRI101 and pRI106 were lost from *Agrobacterium* and *Rhizobium* transciplants only upon release of selection.

3.2.5. Mobilization of the deletion derivatives of pCU1 to *Pseudomonas stutzeri* JM300

The following deletion derivatives of pCU1 were tested for their transfer/mobilization to *P. stutzeri* JM300: pCU8, pCU10, pCU11, pCU13, pCU16, pCU19, pCU28, pCU31 and pCU33. The genetic regions of pCU1 contained in these derivatives are shown in Fig. 3.4. Except pCU8 and pCU19, all other derivatives have lost at least a part of the *tra* region of pCU1 and are hence, non-self-transmissible. However, because they contain the *oriT* of pCU1, they can be mobilized when pCU101 is provided *in trans* (Table 3.4). pCU101 is a clone of the complete *tra* region of pCU1 in plasmid pACYC184 (Thatte and Iyer, 1983). Table 3.4 shows the transfer/mobilization frequencies of the deletion derivatives of pCU1 in *E. coli*-*E. coli* and *E. coli*-*P. stutzeri* JM300 mating experiments. Notably, pCU101 could not be recovered in JM300 (transfer frequency $<10^{-9}$). Also, pCU1 was retransferred back from *P. stutzeri* JM300 to *E. coli* C600 (Nal^r) at a frequency of 10^{-4} .

Table 3.3. Maintenance of pCU1 and pCU785 in the transipients

Species	% cells retaining ^a	
	pCU1	pCU785
<i>Agrobacterium tumefaciens</i> ^b	0	0
<i>Aeromonas hydrophila</i>	37.5	27.7
<i>Alcaligenes faecalis</i>	NA ^c	89.1
<i>Bradyrhizobium</i> ^b sp.	0	0
<i>Enterobacter aerogenes</i>	92.3	94.1
<i>Pseudomonas stutzeri</i> JM300	0	NA
<i>Pseudomonas putida</i>	NA	84.1
<i>Rhizobium meliloti</i> ^b	0	0
<i>Salmonella enteritidis</i>	93.3	90.1
<i>Serratia liquefaciens</i>	25.5	79.1

^aThe numbers are averages of three determinations and indicate percentage of cells expressing plasmid-encoded antibiotic marker(s) following release of selection.

^bFrequency in 10⁶ cells. In other cases, frequency in 3 x 10² cells.

^cNA, Not applicable.

Fig. 3.4. Map of the deletion derivatives of pCU1 tested for their transferability to *Pseudomonas stutzeri* JM300. Plasmid regions present in the deletion derivatives with reference to the map of pCU1 are shown. Sizes of the plasmids (in kb) are: pCU1, 39; pCU8, 37; pCU10, 28.8; pCU11, 27.8; pCU13, 28.8; pCU16, 36.7; pCU19, 33; pCU28, 22.8; pCU31, 17.6; pCU33, 22.8. The genetic symbols of pCU1 are as described in Fig. 1.1. The shaded area represents the region of pCU1 inferred to be required for the establishment of the plasmid in *P. stutzeri* JM300.

Table 3.4. Frequency of transfer of the deletion derivatives of pCU1 to
Pseudomonas stutzeri JM300

Derivative	Transfer frequency ^a
pCU8	1.1×10^{-8}
pCU10 ^b	$< 1 \times 10^{-9}$
pCU11 ^b	$< 1 \times 10^{-9}$
pCU16	1.4×10^{-8}
pCU19	1.1×10^{-8}
pCU28 ^b	$< 1 \times 10^{-9}$
pCU31 ^b	$< 1 \times 10^{-9}$
pCU33 ^b	$< 1 \times 10^{-9}$

^aIn control experiments transfer of all deletion derivatives between *E. coli* C600 and *E. coli* GS500 was observed (frequency about 10^{-2} - 10^{-4})

^bMobilized by pCU101 *in trans*. pCU101 is a clone of the *tra* region of pCU1 in pACYC184.

3.3. DISCUSSION

Limitations to the host-range of a conjugative plasmid may be because of its inability to be transferred to a bacterial recipient in a mating reaction or its inability to replicate and be stably maintained intracellularly following such a transfer. It is also possible that the host-chromosomal functions contribute to such limitations. Either the plasmid lacks recognition signals to utilize the host products or the host encodes/lacks a function that does not/does permit plasmid maintenance. Mutations in the *E. coli* chromosome that specifically affect plasmid maintenance have been discovered (Biek and Cohen, 1986; 1989).

Representatives of bacteria classified on the basis of rRNA homology (Woese, 1987) were chosen for this study. Those used in this study comprise the so-called Gram-negative purple bacteria. The group includes most of the classically defined Gram-negative genera and is thought to have evolved from a photosynthetic ancestor (a purple bacterial type) (Woese *et al.*, 1985). The group of purple bacteria is further subdivided into α , β , γ and δ subgroups. Table 3.2 provides the classification of bacterial species used in this study according to Woese *et al.* (1985).

The *tra* system of RK2, composed of a set of *trans*-acting *tra* genes and a *cis*-acting *oriT* region has been shown to be promiscuous. To be able to mobilize the replicon of pCU1 into as wide a range of bacterial species as possible, a mini-plasmid of pCU1 carrying the *oriT* of RK2 was constructed (Fig. 3.1). pCU785 was mobilized efficiently when the RK2 *tra* functions were provided *in trans* (in *E. coli* S17-1, Table 3.1).

The *tra* system of pCU1, like that of RK2, exhibits a broad-host-range. It is known to transfer plasmids from *E. coli* to distantly related bacteria such as *Azospirillum*, *Azotobacter*, *Proteus*, *Pseudomonas* and *Rhizobium* sp. (Iyer, 1989). Also, transfer of a hybrid

plasmid, pGS36, containing the replicon of RK2 and the *tra* system of pCU1 to *Acinetobacter*, *Aeromonas*, *Proteus* and *Rhizobium* sp. has been observed (P. Mayers and V.N. Iyer, unpublished data). Taken with this evidence, the inability to recover transipients of either pCU1 or pCU785 in *Acinetobacter calcoaceticus*, *Proteus mirabilis*, *Pseudomonas cepacia*, *Pseudomonas stutzeri* PSZ-1 and *Rhizobium leguminosarum* indicates that the pCU1 replicon does not function in these hosts and it is this inability that acts to limit the host-range of pCU1. This leads to the conclusion that the host range of the *tra* system of pCU1 is less limited than that of its replicon. Similar observations have been reported with both F and RK2. The RK2 *tra* system has been shown to transfer plasmid DNA to *Bacteroides fragilis*, a species in which the RK2 replicon is not maintained (Guiney *et al.*, 1984). The *tra* system of plasmid F can deliver DNA into *Pseudomonas aeruginosa*. The F replicon is however, not maintained in this bacterial species (Guiney, 1982).

The bacterial species in which the pCU1 replicon is unable to function are distributed in the α (*R. leguminosarum*), β (*P. cepacia*) and γ (*A. calcoaceticus*) subdivisions of purple bacteria whereas other species within these subdivisions allow the pCU1 replicon to function. Thus, the host factors that act to limit the host-range of the pCU1 replicon do not follow the phylogenetic classification based on the rRNA similarity. A possibility in this regard is that the bacterial species sharing a common ecological niche may constitute the host-range of a plasmid. The environmental conditions of these bacteria may have selected for genetic functions in these species, that are also utilized by the plasmid for its maintenance. Species of *Escherichia*, *Salmonella* and *Enterobacter* share a broad ecological niche and all of these maintain the pCU1 replicon quite stably. This does not however, imply that the host-range of a plasmid does not exceed those bacteria with a common niche.

This is reflected by the ability of the pCU1 replicon to be maintained in *Aeromonas* and *Serratia* species albeit at a lower efficiency (Table 3.3).

In contrast to some of the above-mentioned results, transipients of RK2 could be recovered from all of the host species of α , β and γ subdivisions (Schmidhauser and Helinski, 1985; this study) except *Oceanospirillum minutulum*. Thus, within the purple bacteria, the host-range of the IncN replicon (as exemplified by that of pCU1) is not as extensive as that of the IncP replicon (as exemplified by that of RK2). Limitations to the host-range of IncN group plasmids was also reported by Tardif and Grant (1980). In their studies, IncN group plasmids N3, pCF290, RM98, R45 and R46 were found to be transferred inefficiently to and maintained unstably in non-enteric bacteria.

The second group of bacteria (*Agrobacterium* and *Bradyrhizobium* sp. and *Rhizobium meliloti*) are those in which transipients of both pCU1 and pCU785 were detected and both the plasmids were lost upon release of selection. Another IncN plasmid RN3 has been reported to be suicidal in *Agrobacterium* sp. (Leemans *et al.*, 1981). As a rule but not always (e.g., *R. meliloti* and pCU785), the transipient frequency of these bacteria for one or both the plasmids was also low (10^{-7} - 10^{-9} per recipient). However, members of this group can be distinguished from those of the first group by the fact that they do allow stable maintenance of the plasmids under circumstances that select for plasmid maintenance. Both the plasmids were lost only on release of selection. This property can be attributed to the pCU1 replicon and not to the other plasmid functions of pCU785 (e.g., *oriT* of RK2 or the antibiotic marker) by the fact that pRI101 and pRI106 are maintained in these bacteria under selective pressure while pRI91 and pRI96 were progressively lost from them even under selection. Thus, the factors in these bacteria that limit host-range must be different from those that limit host-range of the first group.

Bacteria of the family Enterobacteriaceae of the γ subdivision as illustrated here by species of *Enterobacter*, *Salmonella*, *Serratia* and *Aeromonas* species fall into a third group. They maintained both the plasmids stably at various frequencies (Table 3.3). The stability of plasmids was tested as the expression of the plasmid-encoded antibiotic marker(s) after 50 generations of cell growth in the absence of any apparent selection for the plasmid. Plasmid markers or entire plasmids have been reported to insert themselves into the genome of various bacterial species (Chakraborty *et al.*, 1978; Chatterjee and Chakraborty, 1982; Breton *et al.*, 1985). In order to gain further confidence that pCU1 and pCU785 were maintained as extrachromosomal elements in the bacterial species mentioned above, the physical demonstration of the plasmid by agarose gel electrophoresis (for pCU785) and retransfer of the plasmid to *E. coli* (for pCU1) were done. In the latter case, the range of transfer frequency was 10^{-2} - 10^{-4} .

Transcipients of pCU785 and not of pCU1 were obtained in *Alcaligenes faecalis* and *Pseudomonas putida*. The possible explanations of this result are (a) a lower frequency of the transfer of pCU1 to these bacteria, (b) restriction of plasmid DNA upon transfer to the recipient or (c) the presence of DNA sequences in pCU1 (but not in pCU785) that destabilize its intracellular maintenance. Alternatively, *oriT* sequences of RK2 in pCU785 may confer stability to it in these bacteria. Recently, Schmidhauser *et al.* (1989) demonstrated that the *oriT* DNA sequences of plasmid RK2 contribute to the stability of the RK2 replicon in a host-specific manner.

In *P. stutzeri* JM300, transcipients of pCU1 and not those of pCU785 were obtained. A plausible explanation in this case is that additional DNA sequences that are required for the replication of pCU1 in JM300 are present in pCU1 but not in pCU785. An examination of the deletion derivatives of pCU1 for their transferability (or mobilizability) to JM300

identified a 5.9 kb region within the *tra* region of pCU1 (restriction map co-ordinates 33.1-39.0 in the map of pCU1), the presence of which in the deletion derivatives enabled its transfer (or mobilization). It is not known whether this 5.9 kb host-range extension region can act *in trans*. A comparative region in another IncN plasmid pKM101 (the *tra* regions of pCU1 and pKM101 exhibit extensive similarities in their genetic organization, Iyer, 1989) contains a DNA region, *kilB*, which specifies host-lethality in *E. coli*. A 3.1 kb DNA segment containing *trfB*, *korB* and *incP1* was shown to be required for the stable maintenance of RK2 in *A. tumefaciens*, *Caulobacter crescentus*, *R. meliloti* and *Rhodospseudomonas sphaeroides* in addition to the minimal replication determinants of RK2: *oriV* and *trfA* (Schmidhauser and Helinski, 1985). The same authors showed that an additional DNA segment of the plasmid containing the promoter and the *kilD* function of *trfA* was required for plasmid maintenance in *A. calcoaceticus*. Further, transposon Tn7 insertions within the primase gene of plasmid R18 (a plasmid similar to RK2, Burkhardt *et al.*, 1979) affected its transmissibility from *P. aeruginosa* to *P. stutzeri* but not between strains of *P. aeruginosa* (Krishnapillai *et al.*, 1984). The primase gene of plasmid RP4 (similar to R18 and RK2) is part of its conjugative transfer region (Lanka and Barth, 1981). Mutations in the primase gene of RP4 also depressed its transfer to *S. typhimurium* (Willetts and Wilkins, 1984). Similarly, primase-defective mutants of plasmid ColIb-P9~~drd~~-1 transferred at a considerably lower frequency in both *E. coli* and *S. typhimurium* (Chatfield *et al.*, 1982). Primase genes within IncN plasmids have not been described. The role of the above 5.9 kb region with regard to its ability to transfer pCU1 to JM300 remains to be determined.

In conclusion then the mini-derivative of pCU1 (pCU785) exhibits nearly the same host-range as the parental plasmid indicating that the molecular determinants of the host-

range of pCU1 are closely linked to its replicon. Further, the bacterial host-range of pCU1 is wider than those of the plasmids like ColE1, pSC101, F and P1 (Jacob *et al.*, 1977) and it is more limited than that of RK2 (Thomas and Smith, 1987; Thomas and Helinski, 1989) and RSF1010 (Frey and Bagdasarian, 1989).

CHAPTER 4

Structural Organization of the Replicon of pCU1

4.1. INTRODUCTION

From the results presented in the previous Chapter, it was concluded that although some determinants of the host-range of pCU1 are located in regions distant from the replicon region, by and large, the host-range of a mini-derivative containing the replicon of pCU1 (=pCU785) was similar to that of the parental plasmid. This focussed attention on the replicon region and emphasized the need for a detailed characterization of the replicon. The next two chapters which are central to this thesis represent a beginning in this direction.

Kozlowski *et al.* (1987) inferred that a 2 kb DNA region of the 39 kb pCU1 DNA contained the plasmid's replicon. This was based on the ability of this 2 kb DNA fragment to (a) confer *E. coli polA*-independence to pBR329 (a *polA* dependent plasmid) and (b) rescue a Tc^r-encoding DNA fragment originating from a heterologous plasmid, pMK16 when covalently linked to them.

The fact that the 2 kb region bestowed *polA*-independence functions to pBR329 suggests at least two possibilities: (i) that it encodes a gene product that substitutes for the *E. coli* polymerase I protein for its replication or (ii) that the pCU1 replicon adopts a replication mode independent of *E. coli polA* (see also Chapter 5). Since the pCU1 replicon has a relatively broad host-range (Chapter 3) this replicon must either specify most functions for plasmid maintenance in different bacterial species or it must have the ability to recognize and utilize the varied host-encoded gene products for replication in the relevant hosts.

Other plasmid replicons that are independent of *E. coli polA* and that exhibit a broad host-range have been shown to encode replication protein(s) of MW >25,000 (Scott, 1984; see also Chapter 1). From the nucleotide sequence of the 2 kb fragment of pCU1

Kozlowski *et al.*(1987) identified only one ORF, ORF207, that encodes a putative polypeptide of comparable MW. These authors pointed out that this ORF encompasses all members (thirteen 37 bp units) of an iteron family (Group I) and hence, would produce an unusual polypeptide of monotonous amino acids. Also, the transcriptional regulatory sequences of this ORF are not in accordance with those described as consensus *E. coli* promoter signals (Reznikoff and McClure, 1986). The two other smaller ORFs identified from the sequence (ORF48 and ORF57) encode putative polypeptides of much smaller size. The differences between the structural features of the pCU1 replicon and those of the other plasmids is thus, readily apparent. The following questions therefore, arose: is the conclusion of Kozlowski *et al.* that the minimal replication determinants of pCU1 are contained within the 2 kb *Pvu*II fragment valid?, are the extraneous DNA sequences viz., pBR329 and the Tc^r-fragment (of pMK16) respectively providing function(s) for the replication to proceed from the 2 kb region? In this chapter, I show that a plasmid containing only the above-mentioned 2 kb DNA sequences can be obtained and further, that it contains determinants for (i) DNA replication, (ii) stable plasmid maintenance and (iii) expression of plasmid incompatibility.

4.2. RESULTS

4.2.1. Autonomous replication of the 2 kb *Pvu*II fragment as a plasmid

The strategy of this experiment is shown in Fig. 4.1. The source of the 2 kb fragment was pCU45 (Fig. 5.1). Plasmid pCU45 contains the unique 2 kb *Pvu*II fragment of pCU1 cloned into the *Pvu*II site of pBR329. The origin of the 2 kb fragment for the

Fig. 4.1. Outline of the strategy employed to obtain the mini-replicon of pCU1 without any additional DNA sequences. pCU45 (Kozlowski *et al.*, 1987; Fig. 5.1) was the source of the 2 kb *Pvu*II region of pCU1. pCU45 contains the 2 kb region cloned into the unique *Pvu*II site of pBR329. pCU45 was first digested with *Pvu*II and the resulting fragments were separated in a 0.7% agarose gel. The 2 kb fragment was recovered from the gel by the 'Gene-clean' method (Section 2.9). The 2 kb DNA fragment was then self-ligated *in vitro* using T4 DNA ligase and was co-transformed with pACYC184 (a plasmid that encodes resistances to Cm and Tc) into *E. coli* HB101 selecting for Cm^r-transformants. From these, a colony carrying both pACYC184 and the 2 kb as a plasmid (designated pCU725) was identified and regrown in LB without antibiotics to yield a strain harboring only pCU725.

construction of pCU45 was pCU32, which is a deletion derivative of pCU1 (Kozłowski *et al.*, 1987).

Plasmid pCU45 was first digested with *Pvu*II and the products were separated on an agarose gel. The 2 kb fragment was recovered from the gel by the 'Gene-clean' method and self-ligated *in vitro*. The ligation mixture was then used to co-transform *E. coli* HB101 with plasmid pACYC184 (pACYC184 encodes resistances to Tc and Cm; Chang and Cohen, 1978). Transformants were selected on LA containing Cm. Screening of twelve transformants revealed that one of them contained both pACYC184 and possibly the 2 kb fragment as a plasmid. This double-transformant was grown in LB without any antibiotic selection for approximately 65 cell generations and dilutions spread on LA to obtain single colonies. Selection for the maintenance of pACYC184 was thus released in this process. 600 such colonies were replica-plated on LA and LA containing Cm. Of the four Cm^r colonies thus obtained, plasmid analysis showed that two of them contained the 2 kb *Pvu*II DNA of pCU1 as a plasmid. One of these derivatives was named pCU725 (Fig. 4.2). A probe containing the internal DNA of the 2 kb region (nucleotide co-ordinates 1-1315 cloned with a Tc^r-encoding fragment of pBR322 (see Section 5.2.4) hybridized to pCU725 while pBR322 and pACYC184 did not (Fig. 4.3). This demonstrated that pCU725 contained the replicon of pCU1 and that it can exist as an autonomous plasmid.

The very existence of pCU725 as a plasmid demonstrates that it should contain determinants for autonomous replication in *E. coli*. HB101(pCU725) was grown in LB for approximately 40 cell generations and dilutions of this culture were spread on LA plate. Twenty four individual colonies were randomly picked and assayed for the presence of pCU725. This was done by isolating plasmid DNA and using it for electrophoretic analysis in agarose gels. All of them retained pCU725. This showed that pCU725 was stably

Fig. 4.2. Agarose gel electrophoresis of *Pvu*II restricted plasmid DNA preparations from HB101(pCU45), lane b; HB101(pACYC184), lane c; HB101(pACYC184, pCU725), lane d; HB101(pCU725), lane e. Lane a contained 1 kb DNA MW standards (GIBCO-BRL, Anonymous, 1989). Note that only the 1, 1.6, 2, 3, 4 and 5 kb DNA fragments are marked on the left of the gel photograph. Arrow indicates the 2 kb *Pvu*II DNA fragment originating from pCU1.

Fig. 4.3. Hybridization of pCU751 (Section 5.2.4) to pCU725. A is the agarose gel photograph and B is the respective autoradiogram in both the top and the bottom panels. The respective probes are indicated above the autoradiograms. In both the top and bottom panels lane 1 contained 1 kb MW standard DNA; lanes 2, 3, 4 and 5 contained *Pvu*II restricted plasmid DNA preparations from HB101(pCU45), HB101(pACYC184), HB101(pACYC184, pCU725) and HB101(pCU725) respectively. In the top panel, lanes 6 and 7 contained *Eco*RI and *Pvu*II restricted plasmid DNA preparations from HB101(pCU751) and HB101(pBR322) respectively. Note that pCU751 (containing nucleotide co-ordinates 1-1315 of the 2 kb region of pCU1, Table 2.3) when used as a probe hybridized to pCU725 (lane 5 in the autoradiogram in the top panel) but that pBR322 and pACYC184 when used as probes did not (lane 6 in the autoradiogram in the top panel and lane 5 in the autoradiogram in the bottom panel). pCU751 and pBR322 hybridized to the 0.5 kb and 1.6 kb DNA bands of the 1 kb DNA ladder (lanes 1 in the autoradiograms in the top panel) and pACYC184 hybridized to the 0.5 kb DNA band of the 1 kb DNA ladder (lane 1 in the autoradiogram in the bottom panel) because pBR322 DNA sequences are represented in the 0.5 and 1.6 kb DNA fragments of the 1 kb DNA ladder DNA (Anonymous, 1989).

A

B

pCU751 pBR322 : PROBE
1 2 3 4 5 6 7 1 2 3 4 5 6 7

A

B

pACYC184 : PROBE
1 2 3 4 5

inherited as an autonomous replicon in HB101 for at least 40 cell generations without any apparent selection. Next, HB101(pCU725) was transformed with pCU832 (pCU832 is a hybrid of the 2 kb *PvuII* region and DNA fragment of pH79 containing its *Tc^r* gene). Plasmid analysis of twenty transformants showed that all of them had lost pCU725, but now contained pCU832. It should be noted that in this case, selection for the maintenance of pCU832 was imposed and there was no apparent selection for the maintenance of pCU725. Loss of pCU725 in these transformants is inferred as due to the expression of incompatibility between pCU725 and pCU832 (Section 5.2.14).

4.2.2. Isolation of a spontaneous deletion derivative of the 2 kb region

During the construction of pCU725, a derivative smaller than 2 kb (1.7 kb) was also isolated. Restriction analysis of this plasmid (=pCU709-3) showed that the restriction site for *PvuII* was still retained. This indicated that the deletion was probably internal to the 2 kb region. In order to localize the deletion endpoints precisely, pCU709-3 was cloned into the *SmaI* site of M13mp19 after *PvuII* digestion. The derivative that contained the upper strand of the 1.7 kb plasmid (by matching to the upper strand of the 2 kb region as described by Kozlowski *et al.*, 1987) was designated M13BOB5 and that which contained the complementary strand as M13BOB6. DNA sequence analysis of M13BOB6 using the universal primer and primers P1, P2, P3, P4, P5 (Kozlowski *et al.*, 1987) and K2 (Table 2.5) revealed that the deletion had occurred between co-ordinates 1533-1828 (nucleotide co-ordinates as described in the 2 kb sequence, see Fig. 4.5). pCU709-3, like pCU725, was also shown to be stably inherited in HB101 for at least 40 cell generations and to express incompatibility to pCU832.

The pCU1 replicon derived sequences in the RF form of M13BOB6 as an *EcoRI*-*HindIII* DNA fragment was cloned with a Km^r -encoding fragment originating from Tn5 to yield pCU744. pCU744 was shown to be stably inherited in *E. coli* for at least 40 cell generations and also to express incompatibility to pCU832 (Table 5.9). Also, *PvuII* restricted pCU709-3 was cloned into the *PvuII* site of pBR329 within the latter plasmid's Cm^r gene to construct pRI145. As shown in Table 5.3, pRI145 exhibited *E. coli polA*- independence phenotype. These results indicate that a deletion within the Group I iterons did not affect the following features of the pCU1 replicon: (a) autonomous replication, (b) stable inheritance, (c) *E. coli polA*-independence and (d) plasmid incompatibility.

4.2.3. Structural organization of the 2 kb region

During the DNA sequence analysis of M13BOB6, errors in the published nucleotide sequence of the 2 kb region were observed. Therefore, the entire DNA sequence of the 2 kb region was determined again from M13BOB1 using the primers denoted in Table 2.5. M13BOB1 is a clone of the smaller *EcoRI*-*HindIII* fragment of pCU45 within the *EcoRI*-*HindIII* segment of the polylinker of M13mp19 and hence, contains all of the 2 kb region DNA within it. Figs. 4.4a-d are photographs of the relevant sections of the autoradiograms of the DNA sequencing gels to show the corrections made in the published DNA sequence and their respective sites. As a consequence of the corrections it was found that the region is 2053 bp long and its complete DNA sequence is given in Fig. 4.5. Henceforth, this will be referred to as the 2 kb region. A summary of the salient features of this DNA sequence is depicted in Fig. 4.6.

Fig. 4.4. Autoradiograms of the sequencing gels (note that only the relevant sections in which the DNA sequence was found to be different from the previously published sequence of Kozlowski *et al.*, 1987 are shown). The nucleotide positions are the respective coordinates as shown in Fig. 4.5. The revised and original DNA sequences are provided for comparison. The letters G, A, T and C above the respective lanes indicate the respective dideoxynucleotide used in the sequencing reaction mixtures.

(a)

(i)

Nucleotide positions	24-38
Revised sequence:	5'-TTCTGTTTTTCTGAG
Original sequence:	5'-TTCTCTTTTTTCTGAG

(ii)

Nucleotide positions	255-242
Revised sequence:	5'-CAGAACCGCGAAAA
Original sequence:	5'-CAGAACGCCGGA AAA

(iii)

Nucleotide positions	533-520
Revised sequence:	5'-TACATTTTTTACAG
Original sequence:	5'-TACATTTTTTACAG

Fig. 4.4. continued

(b)

(i)

Nucleotide positions	730-717
Revised sequence:	5'-TACTGAAGCTGTGA
Original sequence:	5'-TACTGAAGGCTGTGA

(ii)

Nucleotide positions	849-859
Revised sequence:	5'-GCGTGTTTGAG
Original sequence:	5'-GCGTTGTTTGAG

(iii)

Nucleotide positions	857-871
Revised sequence:	5'-GAGTTAAGCCGTTCT
Original sequence:	5'-GAGTTAAGCCGTTCT

i

ii

iii

Fig. 4.4. continued

(c)

(i)

Nucleotide positions	953-938
Revised sequence:	5'-GAGCTGATCGGCTGCC
Original sequence:	5'-GAGCTGAAGTTGCC

(ii)

Nucleotide position:	1087-1075
Revised sequence:	5'-ACTTTCATAAAGG
Original sequence:	5'-ACTTTCATAAAGG

i

ii

Fig. 4.4. continued

(d)

(i)

Nucleotide positions	1230-1217
Revised sequence:	5'-TCATTTATCTGCTT
Original sequence:	5'-TCATTTATTGCTT

(ii)

Nucleotide positions	1257-1243
Revised sequence:	5'-CTATACTTAGCAAGT
Original sequence:	5'-CTATACTTAGGCAAGT

i

ii

Fig. 4.5. Revised DNA sequence of the 2053 bp *Pvu*II DNA fragment of pCU1 containing its replicon. See Fig. 4.6 for a summary of the salient features deduced from this DNA sequence.

10 20 30 40 50 60 70 80
 CTGTTTCATTCTGTTTCTGAAATCTGTTTCTGAGCATCTGTGGGCTCCGAGTATTTGTTACAGAAAGGATATA
 GACAAAGTAGACAAAGACTTTAAGACAAAAGACTCGGTAGACACCCGAGGCATCAAAACCAATGTCTTCTGATAT
 90 100 110 120 130 140 150 160
 CTCAGAAATAATAGGGGTCAATACAGTACGATTTTATAAACTTTATTTTATTTGAGGGGTAGGCCCGGTGCGGCACGA
 GAGTCTTATTTATCCCCAGTTATGTTTCATGCTAAAATATTTGAAATAAAATAAACTCCGCTCCGGGCCACGCCGTGCT
 170 180 190 200 210 220 230 240
 GCGCGCGCTTGATGGTGGCGAAAGGTGCTGGCGCCATGCTTGGATTAAAACATGAACCGTGAAGAACTGCGAAACTT
 CGCGCGGCAACTACCACGGCGCTTTCCACGGACCGCGGTACGAACCTAATTTGTACTTGGCACTTCTTGACGCTTTGAA
 250 260 270 280 290 300 310 320
 GTTTTCGCGGTTCTGAGCGGTTGACCGAGCGCGAAGTGGTAAGCGATGATATGCACATATCCACAGGCATATTTTAAA
 CAAAGCGCCAAAGACTCCCCAACGGCTCGGGCGCTTCCACATTGCTACTATACGTGTATAGGTGTCGCTATAAAATTT
 330 340 350 360 370 380 390 400
 AGGTATTTTATAGATTTTATCTTTTAAAGTCTTTAGAGCTATATACTCATTGATTTAAATCATAAATAAGTGTT
 TCCATAAAATATCTAAAAATAGAAAAATTTCAAAAAATCTCGATATATTGAGTAACTAAATTTAGTATTTATTCACAA
 410 420 430 440 450 460 470 480
 ATCTCTGGGAATCCGCCACCTTGTATGGGAATTTGGCCACCTATCTATGGGAACACCCACCTTATGGGAATTA
 TAGAGACCTTAGCGGGTGAACAATACCTTAACCGGGTGGATAGATACCTTTGTGGGGTGAATGATACCTTAAT
 490 500 510 520 530 540 550 560
 GCGCACCTTGTATGGGAATTTGGCCACCTTAGACGAAACTGTAAAAATGTATTTACTTGTTTGAACCTTTGTGGTAGTG
 CGGTTGAACAATACCTTAAACCGGGTGAATCTGCTTTGACATTTTACATAAATGAACAACTTGAACACCATCAC
 570 580 590 600 610 620 630 640
 TGGAGAGTAATTTTAAACCCACAAAGGCAAGGCTCATGGATAAGTTGCTGAACAAAAAGTAAAGTTAAGCAGTCTAAC
 ACCTCTCATTAATAAATTTGGGTGTTTCCGTTCCGAGTACCTATTCAACGACTTGTTTTCTATTTCAATTCGTCGATTG
 650 660 670 680 690 700 710 720
 GAGCTTACCGAAGCTGCTTACTACCTCTCGCTAAAAGCAAGCGCGTCTCTGTTATGTCTTATGCAGACGTATTTAC
 CTCGAATGGCTTCGACGAATGATGGAGAGCGATTTTCGTTTCGCGCAAGAGACCAATACGAATACGTCTGCATAAAGTG
 730 740 750 760 770 780 790 800
 AGCTTCAGTAAGCGAAGATGATGATGAGATGGCTGTACTCGGTGACTCTACTTTCAAAGTAAAGGTGGCTGACTATCAGC
 TCGAAGTCATTGCGTTCTACTACTACTCTACCGACATGAGCCACTGAGATGAAGTTTCATTCCACCGACTGATAGTCG
 810 820 830 840 850 860 870 880
 AAATTTTTCAGGTAAAGCGTAACCAAGCTATCAAGGATGTTAAAGAGGCGGTGTTGAGTTAAGCCGTTCTCGGTAATC
 TTTAAAAAGTCATTGCGGCTTGGTCCGATAGTTCCATCAATTTCTTCCGACAAACTCAATTCGGCAAGACGCCATTAG
 890 900 910 920 930 940 950 960
 TTTTACCGAAGAAAGGGAGTTTGAAGTGCCTCGCGCGCCCTGGCTAACAGAGGCTGGCAGCCGATCAGCTCGTGGTAT
 AAAATGGGCTTTCTTCCCTCAAAACTGACGCGCGCGCGGGGACCGATTGTCTCCGACCGTCCGCTAGTCCGACCATTA
 970 980 990 1000 1010 1020 1030 1040
 CTGGAAATCGAATTTAAACATAAACTCCTGCGGTACATTTACGGCTGACGAACAGTTTCCACCTACTCGCTCCGCG
 GACCGTTTAACTTAAATTTGATTTGAGGACGCCATGTAAATGCCGAGTCTTGGTCAAGTGGTGGATGAGCGAGCGCG
 1050 1060 1070 1080 1090 1100 1110 1120
 ATTGTGCGAGTCTTCGAATCCACGGACGATCCGCTTTATGAAAGTCTTGTCTCAATTCAAATCTTCAGGCTTATGGGT
 TAACACCGCTCAGAAGCTTTAGGTGCTGCTAGCGGAATACTTTCAAGACGAGTTAAATTTAGAAAGTCCGAATACCCAA
 1130 1140 1150 1160 1170 1180 1190 1200
 ACTACTCATGCTTGGTTAAATGACCGTTTCTTTTCCCGGAATCCCAACAGAAAGTGGCAGAGTTGAACGATCTTT
 TGATGAGTACGAACCAATTTACTGGCAAGGAAGACGGCTTAGGGTGTCTTCTTGAACCGTCTCAACTTTGCTAGAAA
 1210 1220 1230 1240 1250 1260 1270 1280
 CCTGATCCTGCTCTCAGCAGATAAATGAGAAACACCTTTACTTGTAAATATAGTATTGATGATTGAGGAATAATTC
 GGAAGTAGGACGAGAGTTCTCTATTACTCTTTGTGGAATGAACGATTATATCATAACTACTAAGTCTTTTAAAG
 1290 1300 1310 1320 1330 1340 1350 1360
 TGTCTCAATTAATGATAAGCAAAATCCGCTCTGACATAAATCAGCACATGAGCCTGTCTATTGACAAATTTTGTCA
 ACAAGATTTAATACTATTCTTTAGGGCAGCTGTATTAGTCTGTGTACTCGGACAGTAACTGTTTAAAAACAGT
 1370 1380 1390 1400 1410 1420 1430 1440
 TGAAGATGGCGAATTTCCACACAGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACACGCGACCGCGCGCGCAAGG
 ACTTCTACCCGCTTAAAGGTGTGTGCTGCGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCGGTGGCTGCGCGCGCTTCC
 1450 1460 1470 1480 1490 1500 1510 1520
 TGGCGGATTTCCACACGCGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACACGCGACCGCGCGCGCGCAAGGTGGCG
 ACCCGCGTAAGGGTGTGCGGTGCGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCTGCGCGCGCGCGCGCTTCCACCGG
 1530 1540 1550 1560 1570 1580 1590 1600
 GGATTCCACACGCGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACACAGCACCGCGCGCGCGCAAGGTGGCGGATTT
 CCTAAGGGTGTGCGGTGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCTGCGCGCGCGCGCGCTTCCACCGCGCTAAA
 1610 1620 1630 1640 1650 1660 1670 1680
 CCACACAGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACACAGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACAC
 GGTGTGTGCTGCGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCGGTGCGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTG
 1690 1700 1710 1720 1730 1740 1750 1760
 GGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACACAGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACACAGCAC
 CGGTGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCGGTGCGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCTGCG
 1770 1780 1790 1800 1810 1820 1830 1840
 GGCACCGCGCGCAAGGTGGCGGATTTCCACACAGCACCGCGCGCGCGCAAGGTGGCGGATTTCCACACAGCACCGCGCG
 CCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCTGCGCGCGCGCGCGCTTCCACCGCGCTAAGGGTGTGCGGTGCGCGCGG
 1850 1860 1870 1880 1890 1900 1910 1920
 CGGCAAGGTGGCGGATTTCCATAACTTTAATTATACCTTTGTGTTATTTGTGGATTGTGCAAGTCACTGCGCGCTGCG
 GCGCTTCCACCGCGCTAAGGGTATTGAATTAATATGAAGACACATAAACACCTAACACGTGAGTCAACCCCGCGACCG
 1930 1940 1950 1960 1970 1980 1990 2000
 CGTGAAGTGGCGGTGCTGCGCGCTAACCGCGCGCGCGCGCTAAGTGCAGTACCGCGCGCGCGCAACCGCGCGCGCGCG
 GCACTGCGACCGCACAGGGGCGATTGCGCGCGCGCGCGCGCTAAGTGCAGTACCGCGCGCGCGCGCGCGCGCGCGCGCG
 2010 2020 2030 2040 2050
 GTACCCCGCGCGCACCGCGCGCGCTGCGCGCGCGCGCTGAGGAGCTGCGCGCG
 CATGGCGCGCGCGTGGCGCGGGTGAACGCTGGGGCACCTCTGACACCGCGTC

Fig. 4.6. Structural organization of the replicon of pCU1 based on the DNA sequence presented in Fig. 4.5. The closed arrowheads spanning nucleotides 1367-1862 encompass thirteen 37 bp tandem direct iterons (Group I iterons). The open arrowheads between positions 415-510 include the two non-tandem 30 bp Group II iterons. Note that a part of these iterons is repeated upstream of the second member of this family and the presence of a spacer region inside the Group II region. The regions that can potentially form hairpin-loop structures are not shown in this figure. For the location of these regions see Fig. 1.2. The ORFs predicted by the DNA sequence are shown by arrows (see also Table 4.1). The ORFs are named based on the number of amino acids contained in the respective putative polypeptide encoded by the ORF. The short lines and small arrowheads indicate the location of the GATC sequences and the putative *dnaA* boxes present within the 2 kb region. Restriction sites for *PvuII* are also shown in the figure.

The salient features of the 2053 bp DNA sequence are:

- (a) the presence of two families of direct iterons called Group I and Group II. Group I includes thirteen 37 bp tandemly arranged sequences spanning nucleotide co-ordinates 1367-1861. Some of these repeats are not entirely identical to one another (see Fig. 4.7). The Group II iterons region includes two non-tandem 30 bp directly repeated DNA sequences (nucleotide co-ordinates 415-444 and 481-510) separated by a spacer region and a region (nucleotide co-ordinates 460-479) that is partially homologous to the tail segment of these iterons. A 19 bp sequence partially contained within one of the members of this non-tandem iteron and partially outside it (nucleotide co-ordinates 406-423) is repeated in an inverted fashion on the complementary DNA strand and straddling each member of the Group I iteron (e.g., nucleotide co-ordinates 1399-1417 and 1436-1454). This inverted repeat structure could potentially form intrastrand hairpin loop structures. With the exception of the changes noted in the preceding paragraph and in Fig. 4.4, the features observed here have already been described by Kozlowski *et al.* (1987) and are confirmed in this study.
- (b) Six ORFs, ORF46, ORF239, ORF203, ORF188, ORF68 and ORF245 are predicted from the corrected sequence. The features of these ORFs are summarized in Table 4.1. (Note that only those ORFs that encode putative polypeptides of >40 amino acids are considered here).
- (c) The pentanucleotide sequence 'ACAGG' is represented twice at nucleotide co-ordinates 304-308 and 1340-1336.
- (d) The tetranucleotide sequence 'GATC' is represented four times at nucleotide co-ordinates 945-948, 1069-1072, 1194-1197 and 1205-1208.
- (e) Fourteen putative DnaA binding sites were identified in the 2053 bp DNA sequence. One of them exhibits one mismatch and the rest, two mismatches to the consensus 9 bp

Fig 4.7. Sequences of the thirteen (and one partial member of) Group I iterons (Kozlowski *et al.*, 1987; Fig. 4.5). Asterisks indicate the positions where variation(s) between the members of the repeats can be found. The third member has an additional nucleotide. Repeats 4, 7, 11 and 12 are identical as are repeats 5, 8, 9 and 10. Note that only the upper strand in Fig. 4.5 is shown (nucleotide positions 1367-1862).

```

      ★      ★      ★      ★
TGGGCG A ATT T CCACAC A GCACCG G CGCCCGGCAA GG
TGGGCG G ATT C CCACAC G GCACCG A CGCCCGGCAA GG
TGGGCG G ATT C CCACAC G GCACCG G CGCCCGGCAACGG
TGGGCG G ATT T CCACAC A GCACCG G CGCCCGGCAA GG
TGGGCG G ATT C CCACAC G GCACCG G CGCCCGGCAA GG
TGGGCG G ATT C CCACAC A GCACCG G CGCCCGGCAA GG
TGGGCG G ATT T CCACAC A GCACCG G CGCCCGGCAA GG
TGGGCG G ATT C CCACAC G GCACCG G CGCCCGGCAA GG
TGGGCG G ATT C CCACAC G GCACCG G CGCCCGGCAA GG
TGGGCG G ATT C CCACAC G GCACCG G CGCCCGGCAA GG
TGGGCG G ATT T CCACAC A GCACCG G CGCCCGGCAA GG
TGGGCG G ATT T CCACAC A GCACCG G CGCCCGGCAA GG
TGGGCG G ATT T CCACAC G GCACCG G CGCCCGGCAA GG
TGGGCG G ATT T CCA

```

Table 4.1. Location of the predicted translational start and stop sites of the ORFs within the 2 kb region (see also Figs. 4.5 and 4.6).

ORF	Start site	Stop site
ORF46	471	611
ORF239	596	1315
ORF203	704	1315
ORF188	749	1315
ORF68	1332	1126
ORF245	1863	1126

dnaA box (TTATCCACA) (Fuller *et al.*, 1984). Those sites with greater than two mismatches have been disregarded in this study. The DNA sequence of the putative fourteen *dnaA* boxes identified and their precise locations are displayed in Table 4.2.

4.3. DISCUSSION

Clerget (1984) showed that an approximately 2 kb DNA segment containing the Km^r gene of Tn2351 of plasmid R1 can function as an autonomous plasmid in *E. coli* because of its close linkage to an approximately 140 bp region encoding replication functions. Rella *et al.* (1987) demonstrated that the Tc gene in plasmid RP1 contributed to the stable inheritance of the plasmid under specific conditions. Therefore, the use of antibiotic-resistant markers to ascertain whether a phenotypically unmarked DNA fragment contains autonomous replication and maintenance functions may not always be conclusive. The previous demonstration that the replicon of pCU1 is contained within a unique 2 kb *PvuII* fragment of the plasmid was partly by its covalent linkage to a Tc^r -encoding DNA fragment from plasmid pMK16 to yield pCU359 (Kozlowski *et al.*, 1987). The effect of the extraneous DNA sequences in pCU359 on any replication event driven from the 2 kb region is not known. In the light of the above observations and reservations, it was necessary to demonstrate unambiguously that the 2 kb region of pCU1 without linkage to any other DNA specified sufficient functions to survive as an autonomous plasmid. This was done by the construction of pCU725.

The technique of *in vitro* self-ligation and co-transformation with a marked unstable plasmid (pACYC184 in this case) to show that the unmarked pCU725 contains genetic functions for autonomous replication is potentially adaptable to other similar situations *viz.*,

Table 4.2. Location of the putative *dnaA* boxes within the 2 kb region (see also Figs. 4.5 and 4.6)

Box No.	DNA Sequence(5'->3')	Nucleotide co-ordinates
1	ATATGCACA	290-298
2	ATATCCACA	298-306
3	CTCTCCACA	567-559
4	TAACCCACA	575-583
5	TTATCCATG	603-595
6	TTATGCAGA	702-710
7	TTAACCATA	975-983
8	ATTTCACACA	1374-1382
9	ATTTCACACA	1486-1494
10	ATTTCACACA	1597-1605
11	ATTTCACACA	1745-1753
12	ATTTCACACA	1782-1790
13	ATTTCACACA	1819-1827
14	CAATCCACA	1898-1890

to determine whether a given DNA fragment has autonomous replication abilities. A similar strategy has been adopted by Kretschmer *et al.* (1975) except that these authors used a temperature-sensitive (Ts) plasmid as the marked plasmid.

Isolation of pCU709-3 indicated that a plasmid smaller than pCU725 retained the pCU1 functions for plasmid replication, stable maintenance and incompatibility. The DNA sequences deleted from the "2 kb" region in pCU709-3 included the internal portion of the tandem Group I iterons. This suggests that not all of the iterated sequences constitute part of the minimal replicon. In the IncX group plasmid, R6K, loss of one of the seven 22 bp direct repeats did not affect replication but loss of three or more did (Kolter and Helinski, 1982). The presence of directly repeated DNA sequences within the origin regions of other plasmids has also been demonstrated (e.g. Churchward *et al.*, 1983; Abeles *et al.*, 1984). Whether the number of the repeated units or their relative positions is important for plasmid DNA replication has not been established in such cases (see also Chapter 5).

The internal nature of the deletion in pCU709-3 also rules out a possible role for the largest ORF, ORF245 in the maintenance of the plasmid. The deletion includes the 5' end of the ORF and also renders it out of phase relative to its translational start site.

From the DNA sequence shown in Fig. 4.5 and using ATG as the translational start codon five other ORFs are predicted. The four ORFs encoding putative polypeptides of 46, 239, 203 and 188 amino acids will be read from left to right and two ORFs encoding putative polypeptides of 245 and 68 amino acids will be read from right to left as described in the figure (Fig. 4.5). Their predicted translational start and stop sites are shown in Table 4.1. ORFs 203 and 188 are contained entirely within ORF239 and are completely in frame with it. ORF68 is contained entirely within the C-terminal end of ORF245 and is completely in frame with the larger ORF. The translational start site for ORF46 is located within the

second member of the Group II repeats and ORF46 extends into the N-terminal end of ORF239. ORF46 and ORF239 are however, not in phase with each other (Figs. 4.5 and 4.6). In this sequence analysis translational start codons other than ATG have not been considered. I am aware that other start codons e.g. GTG and TTG (Stormo, 1986; Aoyama *et al.*, 1989) have been recorded in nature. They have not been considered here because of the rareness of their occurrence.

It is possible that (a) ORF46 and ORF239 and (b) ORF245 and ORF68 could be read from the same transcriptional units. Only ORF239 exhibits appropriately placed ribosome binding sites (Shine and Dalgarno, 1974; nucleotide co-ordinates 584-587 and/or 590-592).

Four 'GATC' sequences are identifiable from the sequence of the 2 kb. These may serve as the methylation sites for the *E. coli* Dam protein (Geier and Modrich, 1979, see Chapter 5) and quite notably all of them are clustered within an approximately 200 bp region.

An exceptionally high number (fourteen) of putative DnaA-binding sites were recognized in the pCU1 replicon. Six of these are present within the Group I iterons and are identical to each other. These vary from each of the other eight *dnaA* boxes that also vary between themselves. Noticeably, the *dnaA* boxes are fairly evenly distributed within 2053 bp and present in both strands of the DNA fragment. *dnaA* boxes and 'GATC' sequences have also been identified in the minimal replicons of Rts1 and ColV-K30 (Itoh *et al.*, 1987; Perez-Casal *et al.*, 1989). However, in contrast to other plasmids, the pCU1 replicon displays a greater number of *dnaA* boxes. The relevance of both the *dnaA* boxes and the 'GATC' sequences are examined in Chapter 5.

CHAPTER 5

**Functional Organization
of the Replicon of pCU1**

5.1. INTRODUCTION

The essential determinants of plasmid DNA replication in any replicon can be grouped into those that (a) act only *in cis* to the element that is replicated and (b) can act *in trans*. (a) will include the origin(s) of plasmid DNA replication wherefrom replication is initiated, provides binding sites for *trans*-acting factor(s) as well as the gene product(s) that act obligatorily and/or predominantly *in cis*; in principle, the components of a *cis*-acting region may or may not be clustered (see Chapter 1). The gene product(s) that constitute (b) is as a rule protein(s) [RNA molecules regulate replication in the plasmids ColE1, R1 and pT181, reviewed by Zverev and Khmel, 1987]. As described and discussed before (Chapter 4), all plasmid-specified determinants of replication of the 39 kb plasmid are contained in a <2 kb DNA region. The structural features of this DNA region was also described in Chapter 4. An analysis that attempts to relate functions to the observed structure was undertaken and constitutes this chapter.

5.2. RESULTS

5.2.1. The pCU1 replicon is *E. coli polA* independent

Kozlowski *et al.* (1987) previously showed that the 2 kb *Pvu*II fragment of pCU1 containing its replicon conferred *E. coli polA*-independence to pBR329 when covalently linked to it (plasmid construct pCU45; Fig. 5.1). The *E. coli* strain used in their study was MM383. It was found that our current laboratory stock of MM383 maintained a pBR replicon at a high efficiency at 42°C (64% survival). Therefore, an evaluation of several of the *E. coli polA* strains for the maintenance of a pBR replicon was at first undertaken. As a consequence of this evaluation, two strains C2107 and CJ261 (=SR1672) were chosen

Fig. 5.1. Restriction map of pCU45 (6.2 kb, Fig. 4.2). The shaded area shows the 2 kb *Pvu*II region of pCU1 that was cloned into the unique *Pvu*II site of pBR329 to construct pCU45 (Kozłowski *et al.*, 1987). Abbreviations used: Ap^r and Tc^r, ampicillin and tetracycline resistances respectively; E, H, Hc, N and Pv, restriction sites for *Eco*RI, *Hind*III, *Hinc*II, *Nar*I and *Pvu*II respectively.

for use in this study. C2107 is an *E. coli* C strain carrying the *polA* (*ts*) allele (Krevolin *et al.*, 1985) and CJ261 is an *E. coli* K-12 strain from which the *polA* gene has been deleted (Joyce and Grindley, 1984). CJ261 and SR1672 are apparently identical strains (Sharma and Smith, 1987). Both CJ261 and SR1672 were used in this study.

pCU1, pCU45, pCU832 and pRI103 (pRI103 was constructed by cloning an *EcoRI*-*HindIII* fragment of M13BOB2 containing the replicon of pCU1 into the *EcoRI*-*HindIII* region of pBR322 selecting for Ap^r and Tc^r transformants) could all be maintained stably in CJ261 and SR1672 when transmitted to them by mating or transformation. These plasmids could also be maintained in C2107 at both 30°C and 42°C. Under similar conditions, pBR322, pBR328 and pBR329 could not transform SR1672. pBR322 transformed C2107 efficiently (>800 transformants/10⁷ cells/ug plasmid DNA) at 30°C but the plasmid was lost from the transformants at 42°C (<1% survival). pBR328 and pBR329 transformed C2107 inefficiently or not at all. These results confirm that the replicon of pCU1 is *polA*-independent and that of the pBR plasmids is *polA*-dependent (Kingsbury and Helinski, 1970).

The general strategy used for deletion analysis of the 2 kb *PvuII* region of pCU1 was as follows. A clone of a deletion derivative of the 2 kb region in a pBR- vector was obtained (in a *polA*⁺ strain as described in Sections 5.2.2.1 and 5.2.2.2). Its *polA*-independence status was examined in CJ261 (and/or SR1672) and/or C2107. *polA*-independence was inferred when the derivative was maintained and loss of *polA*-independence when it was not in the above-mentioned *E. coli polA* mutants (at 42°C only in C2107). In the latter circumstance, the ability of the deletion derivative to replicate in SR1672 and/or C2107 (at 42°C) was examined when the entire 2 kb region was also

provided *in trans* in the same cell (on plasmid pCU714). The display of such an ability indicated that the derivative had lost essential *trans*-acting replication function(s) but that it contained all the *cis*-acting replication determinants. The inability of the 2 kb region to rescue such plasmids *in trans* was a demonstration that the deletion derivatives had lost an essential *cis*-acting function(s) for replication.

5.2.2. Deletion analysis of the 2 kb region of pCU1

5.2.2.1. Substrate used: pCU45

pCU45 is a derivative of the 2 kb region of pCU1 specifying its replicon cloned in the unique *Pvu*II site of pBR329 (Kozlowski *et al.*, 1987; Fig. 5.1). The origin of the 2 kb *Pvu*II DNA was pCU32, a deletion derivative of pCU1. The Group I iterons (as described within the DNA sequence of the 2 kb region) are located at the end closer to the *Eco*RI site in pCU45 (see Section 4.2.3). The Group II iterons are located closer to the *Hind*III site in pCU45.

pCU45 was digested with *Eco*RI and treated with exonuclease *BAL*31 at 37°C. Aliquots were removed at 5 sec intervals and the enzyme was heat-inactivated at 65°C. The DNA in the reaction mixtures were end-filled using the four dNTPs and the Klenow enzyme and ligated using T4 DNA ligase. They were then used to transform HB101 selecting for *A*^r transformants. Plasmid DNA was prepared from each of these transformants and the presence of *Pvu*II site(s) in them was examined. The presence of only one restriction site for *Pvu*II was indicative of a deletion involving the 2 kb region of pCU1. If there were two restriction sites for *Pvu*II on the plasmid in any transformant, such derivatives were not analyzed further. The deletion derivatives were mapped using restriction enzymes *Hinc*II,

NarI and *XmnI*. A map of the deletion derivatives obtained with reference to the 2 kb region is shown in Fig. 5.2. The presence of the Group I iteron region in these plasmids was examined by 'Southern' hybridization. The probe used in this case contained DNA sequences including nucleotide co-ordinates 1374-2053 of the 2 kb region (see Fig. 4.5) within M13mp19. Derivatives 45E159, 45E1513, 45E1516, 45E251, 45E252, 45E1524, 45E1523, 45E1521, 45E1520 and 45E1518 (Class B deletions, Fig. 5.3, Table 5.1) hybridized to this probe demonstrating that they contained all or part of the Group I repeat sequences (Fig. 5.3). Deletion derivatives, 45E151, 45E152, 45E156, 45E1510, 45E355 and 45E358 (Class A, Fig. 5.3, Table 5.1) did not hybridize to the probe indicating that they have lost all of the Group I iteron region.

Deletion analysis of the 2 kb region of pCU45 after linearization of the plasmid with *HindIII* was done as described above. In this case, all the transformants were Tc^r because *HindIII* is located within the promoter region of the Tc gene in pCU45. The ability of the different deletion derivatives to replicate in a *polA* mutant strain is shown in Table 5.1. These results show that at least 100 bp near the Group I iterons region and about 200 bp near the Group II iterons region (including the *PvuII* sites on both ends can be deleted without affecting the replication abilities of the 2 kb region. These estimates are based on the restriction mapping of the deletion derivatives using the restriction enzymes for which sites on the pCU45 are shown in Fig. 5.1.

5.2.2.2. Substrate used: Single-stranded forms of M13BOB1 and M13BOB2

The smaller *EcoRI-HindIII* fragment of pCU45 containing the 2 kb *PvuII* region was cloned into the smaller *EcoRI-HindIII* segment of M13mp19 to yield M13BOB1 (see Fig. 5.6 for a restriction map of M13BOB1). In the phage preparations of M13BOB1 the upper

Fig. 5.2. Map of the deletion derivatives of pCU45. The thin dotted lines indicate pBR329 derived sequences in pCU45. Derivatives 45E151, 45E152, 45E156, 45E1510, 45E355 and 45E358 comprise Class A. Derivatives 45E159, 45E1513, 45E1516, 45E251, 45E252, 45E1524, 45E1523, 45E1521, 45E1520 and 45E1518 comprise Class B. Derivatives 45H405 and 45H408 comprise Class C. Derivatives 45H410 and 45H416 comprise Class D (see also Fig. 5.3). The continuous thick lines indicate the 2 kb derived region present in the deletion derivative with reference to the wild-type 2 kb region (in pCU45). The dotted line in the deletion derivatives indicates that the precise endpoints of the deletions are not known. Abbreviations: E, H, Hc, Pv and X, restriction sites for *EcoRI*, *HindIII*, *HincII*, *PvuII* and *XmnI*. Other symbols are as described in Fig. 4.6.

Class

A

B

C

D

Fig. 5.3. Hybridization of M13BOB2MY19 to the deletion derivatives of pCU45. A is the agarose gel photograph and B is the autoradiogram. *Hind*III digested plasmid DNAs were used as the target species. Lane 17 contained 1 kb MW standard DNA.

Lane	Plasmid derivative ^a
1	45E151
2	45E152
3	45E156
4	45E159
5	45E1510
6	45E1513
7	45E1516
8	45E251
9	45E252
10	45E355
11	45E358
12	45E1524
13	45E1523
14	45E1521
15	45E1520
16	45E1518
18	pCU45
19	pBR329
20	M13mp19

^aSee Fig. 5.2 for a map of the deletion derivatives of pCU45

A **B**

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

Table 5.1. The ability or otherwise of the various deletion derivatives obtained from a deletion mutagenesis of pCU45 (Section 5.2.2.1) to be maintained in C2107 at 42°C

Plasmid ^a	Maintenance ^b
pCU45	+
Class A	-
Class B	+
Class C	-
Class D	+

^aFor the list of plasmids included under different classes and a map of them see Fig. 5.2. All plasmids were introduced into C2107 by transformation at 30°C.

^bIn each case, at least 100 transformants were replicated on LA plates and incubated at 30°C or 42°C (to ensure that the strain itself was not Ts for growth) and on the appropriate antibiotic-containing LA plates which were incubated at the same two temperatures. Results are presented as + or - because all or none of the replicated colonies grew on the antibiotic plates at 42°C.

strand of the 2 kb region (as described in Fig.4.5) was present. M13BOB2 is a clone of the 2 kb *PvuII* region cloned into the *SmaI* site of M13mp19. M13BOB2 extruded the bottom strand of the 2 kb region (Fig. 4.5) in phage preparations along with the M13mp19 sequences. The Group I iteron DNA sequences are closer to the *EcoRI* end in M13BOB1 and the Group II iterons are closer to the *EcoRI* end in M13BOB2 respectively (as determined by DNA sequence analysis of the recombinant M13 bacteriophages produced by M13BOB1 and M13BOB2 using the universal primer).

T4 DNA polymerase-mediated deletions within the 2 kb region were obtained in both M13BOB1 and M13BOB2 in both directions. The limits of the 2 kb region contained in them were ascertained by DNA sequence determinations using the universal primer and the '-40' primer (Table 2.5). About 100-150 bases of the DNA sequence were determined from the respective ends in each of the deletions. The deletion derivatives of progressively smaller size from both ends of the *PvuII* sites of the 2 kb region were identified by their faster mobility during agarose gel electrophoresis in comparison to either M13BOB1 or M13BOB2. The replicative forms of the selected bacteriophage DNAs were then isolated and the sequences derived from the 2 kb were cloned into pBR322, pBR328 or pBR329 as an *EcoRI-HindIII* or as an *EcoRI-PvuII* fragment. A summary of these deletion derivatives is included in Fig. 5.4 and Table 5.2. Results of the experiments examining their ability to rescue the pBR replicon are shown in Tables 5.3 and 5.4. The smallest derivative that exhibited this ability in C2107 was pRI120 containing the DNA sequences 1-1315 of the 2 kb region (Fig. 5.4). However, pRI120 could not transform the *E. coli polA* null strains CJ261 and SR1672 (Table 5.4).

Fig. 5.4. Summary of the salient features of the DNA sequence of the 2 kb region and endpoints of the deletion derivatives of the 2 kb region and subclones of them obtained as described in Sections 5.2.2.2, 5.2.3, 5.2.4, 5.2.5 and 5.2.6 (see also Table 5.2). The designation of the various plasmid derivatives is shown on the right. The numbers above each of the plasmid derivatives indicate the limit of the 2 kb DNA present in the plasmid. The other symbols are as described in Fig. 4.6.

pCU725, pCU714, pCU832
 pCU709-3, pCU744, pRII45
 pCU773, pRII33
 pCU752, pRII28
 pCU751, pRII20
 pRII29
 pRII04
 pRII05
 pRII23
 pRII14, pRII15
 pRII25
 pRII19

Table 5.2. Summary of the salient features of the deletion derivatives of the 2 kb region the deletion endpoints of which have been defined by DNA sequencing (Sections 4.2.2, 5.2.2.2 and 5.2.3)

Plasmid	Restriction fragment used for the cloning	Vector	Nucleotide co-ordinates as in Fig. 4.5.	Antibiotic markers ^a
pCU744	<i>EcoRI-HindIII</i>	-	1-1532; 1829-2053	Km
pRI104	<i>EcoRI-HindIII</i>	pBR322	947-2053	Ap
pRI105	<i>EcoRI-HindIII</i>	pBR322	1375-2053	Ap
pRI114	<i>EcoRI-XmnI</i>	pBR329	1-1016	Ap, Tc
pRI115	<i>HindIII</i>	pACYC184	1-1016	Cm
pRI119	<i>EcoRI-XmnI</i>	pBR329	1-1016	Ap, Tc
pRI120	<i>EcoRI-PvuII</i>	pBR328	1-1315	Ap, Tc
pRI123	<i>EcoRI-HindIII</i>	pBR322	1825-2053	Ap
pRI125	<i>EcoRI-XmnI</i>	pBR329	1017-1315	Ap, Tc
pRI128	<i>EcoRI-PvuII</i>	pBR328	1-1593	Ap, Tc
pRI129	<i>EcoRI-HindIII</i>	pBR322	542-2053	Ap
pRI133	<i>EcoRI-HindIII</i>	pBR322	296-2053	Ap
pRI145	<i>PvuII</i>	pBR329	1-1532; 1829-2053	Ap, Tc

^aAp, Cm, Km and Tc indicate resistances to ampicillin, chloramphenicol, kanamycin and tetracycline respectively.

Table 5.3. The ability or otherwise of the various constructs containing different parts of the 2 kb *Pvu*II region (deletion derivatives and subclones in the pBR- plasmids) to be maintained in the *polA* (*ts*) strain C2107 at 42°C

Plasmid ^a	Maintenance
pCU1	+
pCU45	+
pCU714	+
pCU744	+
pCU751	+
pCU752	+
pCU832	+
pRI103	+
pRI104	-
pRI105	-
pRI114	-
pRI119	-
pRI120	+
pRI123	-
pRI125	-
pRI128	+
pRI129	-
pRI133	+
pRI145	+

^aFor the nucleotides from the 2 kb region present in the plasmids see Fig. 5.4. With the exception of pCU1 which was transferred into C2107 by bacterial mating, all other plasmids were transformed into C2107 by transformation at 30°C. The other procedures were as described in the footnote to Table 5.1.

Table 5.4. Transformation of the strain carrying the *polA* deletion (CJ261) and of the otherwise isogenic strain carrying the wild-type *polA* allele (CM4722)

Plasmid ^a	Ability of the plasmid to transform ^b	
	CM4722(SR1758)	CJ261(SR1672)
pCU45	+	+
pCU751	-	-
pCU752	+	+
pCU832	+	+
pRI103	+	+
pRI104	+	-
pRI105	+	-
pRI114	+	-
pRI119	+	-
pRI120	+	-
pRI123	+	-
pRI125	+	-
pRI128	+	+
pRI129	+	-
pRI133	+	+
pRI145	+	+
pBR322, pBR328, pBR329	+	-

^aFor the nucleotides of the 2 kb region present in these plasmids see Fig. 5.4.

^bAll four strains were transformed. The results for the pairs of strains indicated together were very similar. Note that these pairs are apparently identical. + : 100-150 transformants/0.7 ug of plasmid DNA/10⁷ cells, - : <1 transformant/0.7 ug of plasmid DNA/10⁷ cells.

5.2.3. Construction and properties of plasmids pRI125, pRI114, pRI115 and pRI119

M13BOBA23 is the deletion derivative of M13BOB1 that was used in the construction of pRI120. It contains nucleotide co-ordinates 1-1315 of the 2 kb region in M13mp19. There is a single restriction site for *Xmn*I within the 2 kb region at nucleotide co-ordinate 1016. Two recognition sites for *Xmn*I are also present elsewhere on the M13mp19 genome. *Xmn*I cleavage of DNA yields blunt-ended products. Restriction of M13BOBA23 with the enzymes *Eco*RI and *Xmn*I produced a 0.3 kb DNA fragment that includes nucleotides 1017-1315 of the 2 kb region. This was cloned into the smaller *Eco*RI-*Pvu*II region of pBR328 to construct pRI125 (Fig. 5.5).

*Eco*RI-*Xmn*I digestion of the replicative form of M13BOB1 resulted in three fragments: (a) a 2.3 kb DNA fragment bordered by blunt ends and that is entirely composed of M13mp19 DNA sequences, (b) a 2.4 kb DNA fragment bordered on one side by an overhang created by *Eco*RI and on the other side blunt-ended and composed of nucleotides 1017-2053 of the 2 kb region and (c) a 4.6 kb DNA fragment bordered by blunt ends and that contains nucleotide co-ordinates 1-1016 of the 2 kb region (Fig. 5.6) and part of the M13mp19 DNA sequences. The DNA fragment described in (c) above was isolated and cloned into the *Pvu*II site of pBR328 to yield pRI114. Subcloning of the smaller *Hind*III fragment of pRI114 into pACYC184 resulted in the recombinant plasmid pRI115. In the process, all of the M13mp19 DNA sequences contained in pRI114 described in (c) above have been deleted in pRI115. Thus, pRI115 contains only nucleotide co-ordinates 1-1016 of the 2 kb region (Fig. 5.6). All three recombinant plasmids pRI125, pRI114 and pRI115 were found to exhibit a *polA*-dependent phenotype (Tables 5.3 and 5.4).

pCU752 (Section 5.2.6) contains DNA sequences of nucleotide co-ordinates 1-1593 of the 2 kb region (Fig. 4.5) cloned as an *Eco*RI-*Pvu*II fragment with the *Tc^r* gene of

Fig. 5.5. Construction of pRI125. Sizes of the plasmids (in kb) are: M13BOB1A23, 8.5; pBR329, 4.2; pRI125, 4.5. The shaded region represents part of the 2 kb region derived from pCU1. Abbreviations: Ap^r, Cm^r and Tc^r, ampicillin, chloramphenicol and tetracycline resistances respectively; E, H, Ps and Pv, restriction sites for *Eco*RI, *Hind*III, *Pst*I and *Pvu*II respectively, E+X and E+Pv, double digests with restriction enzymes *Eco*RI and *Xmn*I and *Eco*RI and *Pvu*II respectively; Pv-X, indicates that as a result of cloning restriction sites for both *Pvu*II and *Xmn*I were lost.

Fig. 5.6. Construction of plasmids pRI114 and pRI115. Sizes of the plasmids (in kb) are: M13BOB1, 9.2; pBR329, 4.2, pRI114, 8.8, pACYC184, 4, and pRI115, 5.2 Abbreviations used: same as in Fig. 5.5 except that H, Pv and X also indicate restriction enzyme digestions with *Hind*III, *Pvu*II or *Xmn*I respectively.

pBR322. The smaller *EcoRI-XmnI* fragment of pCU752 containing nucleotide co-ordinates 1017-1593 of the 2 kb region was cloned into the *Cm^r* gene of pBR329 to yield pRI119 (Fig. 5.7). As shown in Tables 5.3 and 5.4, pRI119 exhibited the *E. coli polA*-dependent mode of plasmid maintenance.

5.2.4. Nucleotide sequences of the 2 kb region sufficient to specify autonomous replication

As shown in Table 5.3, pRI120, which contains only nucleotides 1-1315 of the pCU1 replicon (Fig. 4.5) linked to pBR328 is able to replicate in C2107 at 42°C. A reasonable explanation is that this occurs because of the replication of this plasmid is by a mechanism independent of a functional DNA polymerase I. Do the nucleotide co-ordinates of 2 kb present in pRI120 (viz., co-ordinates 1-1315) encode sufficient function(s) for autonomous replication? To address this question, the DNA sequences 1-1315 in an *EcoRI-PvuII* fragment were cloned with the smaller *EcoRI-PvuII* 2 kb fragment of pBR322 encoding *Tc^r* to obtain pCU751. This *Tc^r*-encoding DNA fragment of pBR322 does not contain this plasmid's replicon. pCU751 was found to be stably inherited in *E. coli* HB101. The construction of pCU751 confirmed that all essential determinants of the pCU1 replicon are contained within a 1.3 kb DNA fragment (nucleotide co-ordinates 1-1315 of the 2 kb region, Fig. 5.4). pCU751 could transform C2107 at 30°C and was also maintained in it at 42°C. It is inferred that the region determining *polA*-independence and autonomous maintenance in some wild-type strains (e.g. HB101) is contained within nucleotide sequences 1-1315. However, pCU751 could neither transform CJ261 (SR1672) nor its isogenic *polA⁺* relative CM4722 (SR1758). Apparently, there is an unspecified genetic difference(s) between the two wild type strains HB101 and CM4722. The basis for this difference remains to be investigated.

Fig. 5.7. Construction of pRI119. Sizes of the plasmids (in kb) are: pCU752, 3.5; pBR329, 4.2; pRI119, 4.8. Abbreviations used: same as described in Fig. 5.5.

5.2.5. Construction and properties of pCU773

Plasmid pRI133 is *polA*-independent (Tables 5.3 and 5.4). The nucleotide co-ordinates of the 2 kb region contained in this recombinant plasmid are 296-2053. This DNA segment as a *Pvu*II fragment from pRI163 (see Fig 5.8 for the construction of pRI163) was cloned with a 1.1 kb *Pvu*II fragment from pLG338 that specifies Km^r to yield pCU773 (Fig. 5.8). Thus, the only replicon present in pCU773 is derived from nucleotide co-ordinates 296-2053 of the 2 kb region. This, taken with the results of pCU751 suggests that the replication determinants of the 2 kb region (and hence of pCU1) are contained within an approximately 1 kb contiguous DNA segment of the 2 kb region including co-ordinates 296-1315. It must be pointed out here, that this region excludes all members of the Group I iteron family. Also, the 5' domains of both ORF68 and ORF245 are deleted in pCU751. This means that the Group I repeat sequences and the two ORFs (ORF68 and ORF245) do not constitute part of the minimal replication determinants of the 2 kb region (and therefore, of pCU1).

5.2.6. pCU751 is a copy number mutant

It was observed that the yield of plasmid DNA from HB101(pCU751) was higher than that from a comparable number of cells of HB101(pCU832) and HB101(pCU714) (Fig. 5.9) [pCU714 is a clone of the 2 kb region as an *Eco*RI-*Hind*III fragment from pCU45 with the Km^r gene originating from Tn5]. This suggested that pCU751 is probably maintained at a higher copy number than the 2 kb replicon. In order to examine whether any DNA sequences between nucleotide co-ordinates 1316-2053 (that are absent in pCU751) contributed to the plasmid copy number determination of the 2 kb plasmid (=pCU714), pCU752 was constructed.

Fig. 5.8. Construction of pCU773. Sizes of the plasmids (in kb) are: pUC18, 2.7; pRI133, 6; pRI163, 4; pLG338, 7.3; pCU773, 3. Abbreviations and symbols used: same as described in Figs. 3.1 and 5.5 except that E+H indicates double digests with the restriction enzymes *EcoRI* and *HindIII*.

The smaller *EcoRI-PvuII* fragment of pRI128 (containing nucleotide co-ordinates 1-1593 of the 2 kb region) was cloned with the 2 kb *EcoRI-PvuII* DNA fragment of pBR322 specifying *Tc^r* to obtain pCU752. The copy number of pCU752 was comparable to that of pCU714 (as evident from the comparable yield of plasmid DNA from comparable numbers of cells carrying the respective plasmid, see Fig. 5.9).

The degree of *Tc^r* expressed by HB101(pCU751) was at least two times higher than that expressed by HB101(pCU832) and HB101(pCU752) (Table 5.5). These experiments provide evidence to the notion that nucleotide co-ordinates 1316-1593 contribute to the copy number control of the 2 kb plasmids (pCU832 and pCU714) and pCU752. Further and as shown in Fig. 5.9, the 1374-2053 DNA sequence of the 2 kb region cloned in pBR322 (plasmid pRI105), when present *in trans* could reduce the copy number of pCU751.

5.2.7. Localization of *cis*-acting region(s)

Among the deletion derivatives of the 2 kb region depicted in Fig. 5.4, none shown below pRI120 can be maintained in C2107 at 42°C. They exist in the wild type strain, HB101 and in C2107 at 30°C by virtue of being covalently linked to one of the pBR replicons. Such a loss of maintenance ability can arise because essential gene(s), the product of which can act *in trans* has been deleted. Alternatively, the region(s) that is deleted could be *cis*-acting. To distinguish between these two classes of deletion derivatives, they were transformed into C2107(pCU714) at 30°C and at least 100 transformants were replica-plated on media selecting for the deletion derivatives and were grown at 42°C. The results of these experiments are presented in Table 5.6. The pBR derivatives containing different parts of the 2 kb replicon fall into a class that can be rescued by pCU714 in C2107 at 42°C (pRI104, pRI129) and a class that cannot be rescued (pRI105, pRI114, pRI119 and

Fig. 5.9.

A: Yield of plasmid DNA from approximately 10^8 cells of HB101(pCU751), lane 3; HB101(pCU832), lane 4; HB101(pCU752), lane 5; HB101(pCU714), lane 6. Total plasmid DNA that was isolated in each case was dissolved in 30 μ l of TE buffer and 5 μ l was used for restriction enzyme digestion with *Eco*RI in a 15 μ l volume. All of the reaction mixture was loaded in the respective lanes and electrophoresed. Duplicate experiments are shown. Lane 1 contained 1 kb DNA MW standards.

B: Yield of plasmid DNA from approximately 10^8 cells of HB101(pBR322), lanes 2 and 3; HB101(pRI105), lanes 4 and 5; HB101(pCU751), lanes 6 and 7; HB101(pRI105, pCU751), lanes 8 and 9 and HB101(pBR322, pCU751), lanes 10 and 11. Total plasmid DNA that was isolated in each case was dissolved in 30 μ l of TE buffer. Restriction enzyme digestion and electrophoresis were done as described above. Duplicate experiments are shown. Lanes 1 and 12 contained 1 kb DNA MW standards. Arrowheads indicate pCU751 DNA. Note that the yield of pCU751 DNA was reduced when pRI105 was co-resident in the cell but not when pBR322 was co-resident.

A **B**

Table 5.5. Growth of *E. coli* HB101 carrying the respective test plasmid on LA containing different concentrations of the antibiotic tetracycline (Tc)

Test plasmid	Concentration of Tc in ug/ml						
	20	40	50	60	80	90	100

pBR322	+	+	+	-	-	-	-
pCU832	+	+	-	-	-	-	-
pCU751	+	+	+	+	+	+	-
pCU752	+	+	+	-	-	-	-

Table 5.6. Fraction of C2107(pCU714) transformants^a that maintained the test plasmid at 42°C

Test plasmid	Fraction (%)
pRI104	55
pRI105	11
pRI114	.
pRI115	-
pRI119	-
pRI123	-
pRI125	-
pRI129	71
pBR322	10
pBR328	-
pBR329	-
pACYC184	2

^aAntibiotic selection was imposed for the maintenance of both pCU714 and the test plasmid. At least 100 transformants were tested in each case. Replica plating experiments were done as described in the footnote to Table 5.1.

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pRI125). While pCU714 is stably maintained in C2107 at 42°C, it could not rescue the pBR replicon (pBR322, pBR328 and pBR329) in C2107 at 42°C. These results suggest that the *cis*-acting function(s) of the 2 kb fragment is provided by a region shared by pRI104 and pRI129 and which is also shared by pCU751 (because pCU751 is an autonomous replicon and hence, must contain the minimal *cis*-acting and *trans*-acting functions). In contrast neither pRI114 nor pRI125 was rescued under the same conditions when pCU714 was present *in trans* (Table 5.6). A 368 bp DNA region (nucleotide co-ordinates 947-1315, see Fig. 4.5) can thus, be implicated as specifying the *cis*-acting function(s) of plasmid DNA replication.

5.2.8. Mapping of the essential *trans*-acting region for autonomous replication of the 2 kb region

The minimal determinants of replication of the pCU1 replicon as inferred in Section 5.2.5 are contained between nucleotide co-ordinates 296-1315 of the 2 kb DNA sequence (Fig. 5.4). This rules out a role for either of the two ORFs, ORF68 and ORF245, in plasmid replication. A plasmid (pCU773) containing the regions 296-2053 has autonomous replication abilities. However, a 244 bp deletion of this on the left end (see Fig. 5.4) renders the plasmid (pRI129) dependent on the pBR replicon for survival in *E. coli* HB101 and exhibits a *polA*-dependent phenotype in both SR1672 and in C2107 (at 42°C in C2107). pRI129 can nevertheless, be rescued *in trans* by pCU714 (Section 5.2.7). This indicates that pRI129 lacks a *trans*-acting function(s) specified by the 2 kb region and which is required for autonomous replication. The deleted region in pRI129 contains the Group II iteron region and the N-terminal half of ORF46. It is unlikely (because of its unusual features) that the Group II repeat sequences themselves encode a *trans*-acting gene product. ORF46

then, is the candidate gene that is lacking in pRI129. Attention is drawn to the fact (Fig. 5.4) that pRI129 contains the ORF239, ORF203 and ORF188 regions. Of these, only ORF239 exhibits canonical putative ribosome binding sites (Section 4.2.3). The presence of these ORFs is insufficient to provide the *trans*-acting functions. It is therefore, likely that the region containing ORF46 constitutes a region that specifies at least one *trans*-acting factor for plasmid replication.

5.2.9. Polypeptides encoded by the 2 kb region

In order to determine whether the ORFs predicted from the DNA sequence are functional, the ability of the 2 kb region by itself (plasmid pCU725, Section 4.2.1), a clone of it in pBR329 (plasmid pCU45, Kozłowski *et al.*, 1987; Fig. 5.1) and its deletion derivatives (pCU709-3, pCU751 and pCU752) to encode polypeptides was examined. Two procedures were used: (a) the mini-cell system and (b) an *in vitro* plasmid DNA directed protein synthesis system. The results using the mini-cell system (Fig. 5.10) suggest that the plasmids carrying the 2 kb region (pCU45, pCU1 and pCU832; lanes 1, 2 and 5 respectively) produce a polypeptide of MW 27,000. From lane 3 (pACYC184 without the 2 kb region), a conclusion that this polypeptide is absent cannot be conclusively made. However, a direct visualization of the autoradiogram showed no evidence of such a polypeptide as being encoded by pACYC184. With pJHN11 (lane 4) a polypeptide of MW 27,000 is seen. This is consistent with it being a product of the Km gene (Shingler and Thomas, 1984). In view of the inconclusive observations, the *in vitro* system was used. Results shown in Figs. 5.11 and 5.12 demonstrate that at least two polypeptides of apparent MW 27,000 and 5500 can be attributed as being synthesized from the DNA sequence of the 2 kb region. Since pCU751 is capable of directing synthesis of both these polypeptides, it can be inferred that

Fig. 5.10. Polypeptides synthesized in minicells produced from DS410(pCU45), DS410(pCU1), DS410(pACYC184), DS410(pJHN11) and DS410(pCU832). Ap, Cm and Tc indicate the proteins encoded the DNA sequences specifying Ap^r, Cm^r and Tc^r respectively. The arrow indicates the MW 27,000 polypeptide encoded by the replicon of pCU1 (see also Figs. 5.11 and 5.12). Numbers on the left show the MW ($\times 10^3$) of the markers used. It should be noted that the protein specified by the Km^r gene in pJHN11 migrates at the same position as the MW 27,000 polypeptide because of comparable MW (Shingler and Thomas, 1984; Kornacki *et al.*, 1984).

Fig. 5.11. Polypeptides synthesized *in vitro* from the DNA preparations of plasmids pBR329, pCU45, pCU725 and pCU709-3. Ap and Cm indicate proteins synthesized by the DNA sequences encoding Ap^r and Cm^r respectively. The arrows indicate the two polypeptides of apparent MW 27,000 and 5500 encoded by the replicon of pCU1. Numbers on the left indicate MW ($\times 10^3$) of the marker proteins. Note that the Tc^r gene-encoded protein was not resolved in this gel.

Fig. 5.12. Polypeptides synthesized *in vitro* from the DNA preparations of plasmids pAT153, pBR329, pCU45, pRI103, pCU751 and pCU752. Ap and Cm indicate proteins synthesized from the DNA sequences encoding Ap^r and Cm^r respectively. The big arrow represents the MW 27,000 and the small arrow the MW 5500 polypeptides respectively encoded by the replicon of pCU1. Numbers in the centre indicate the MW ($\times 10^3$) of the markers used. Note that the Tc^r gene-encoded protein was not resolved in this gel.

they are most likely the products of ORF239 and ORF46 respectively.

5.2.10. Point mutations in the 2 kb region

Site-directed polypeptide chain terminating mutations were introduced into ORFs 48 and 57 (based on the earlier published DNA sequence of the 2 kb region, Kozłowski *et al.*, 1987; Fig. 1.2) by P. Fobert, U. Seitzer and V.N. Iyer (unpublished data). As a consequence of the corrections noted in the DNA sequence of the 2 kb region (Section 4.2.3), (a) one of the mutations introduced in ORF48 is mapped at the N-terminal end of ORF239 and the same mutation can be mapped within the C-terminal end of ORF46 (of the revised DNA sequence of the 2 kb region) where it does not introduce any change at the amino acid level and (b) the second mutation introduced into ORF57 now mapped within ORF239. Both these mutations introduce stop codons in the reading frame of ORF239 (Fig. 5.13). A summary of the wild type DNA sequence, its predicted amino acid sequence, the mutant DNA sequence and the mutant amino acid sequence of the two mutations is provided in Fig. 5.14. Both these mutations individually converted the *polA* independent 2 kb replicon to a *polA*-dependent status. The 2 kb region containing the respective mutations as *EcoRI-HindIII* fragments were cloned into pBR329 to yield pCU719 (carrying mutation I) and pCU720 (carrying mutation II) respectively. Neither pCU719 nor pCU720 could transform CJ261 and CJ261(pCU1) (Table 5.7).

Fobert and Iyer (unpublished data) have demonstrated that the 2 kb region alone with either of these mutations can be linked to a Km^r -encoding DNA fragment to yield pCU717 (carrying mutation II) and pCU718 (carrying mutation I). pCU717 and pCU718 exhibited autonomous replication abilities in *E. coli* CC118 and Q125 (*pol*⁺ *su*⁰, strains, Manoil and Beckwith, 1985; D'Ari *et al.*, 1975). Neither of these plasmids could transform

Fig. 5.13. Predicted amino acid sequence of ORF239 (nucleotide co-ordinates 596-1315 as described in Fig. 4.5). Arrowheads indicate location of the two polypeptide chain-terminating mutations introduced into the reading frame of ORF239 (see also Fig. 5.14).

V
 ATG GAT AAG TTG CTG AAC AAA AAG ATA AAA GTT AAG CAG TCT AAC GAG CTT ACC GAA GCT 60
 Met Asp Lys Lys Leu Leu Asn Lys Lys Ile Lys Val Lys Gln Ser Ser Asn Glu Leu Thr Glu Ala
 120
 GCT TAC TAC CTC TCG CTA AAA GCA AAG CGC GGT CTC TGG TTA TGT CTT ATG CAG ACG TAT
 Ala Tyr Tyr Leu Ser Leu Lys Ala Lys Arg Val Leu Tip Leu Cys Leu Met Gln Thr Tyr
 180
 TTC ACA GCT TCA GTA AGC GAA GAT GAT GAT GAT GCT GCT GTA CTC GGT GAC TCT ACT TTC
 Phe Thr Ala Ser Val Ser Glu Asp Tyr Gln Gln ATT TTT CAG GTA AGC CGT AAC CAG GCT ATC AAG
 240
 AAA GTA AAG GTG GCT GAC TAT CAG CAA ATT TTT CAG GTA AGC CGT AAC CAG GCT ATC AAG
 Lys Val Lys Val Ala Asp Tyr Gln Gln Ile Ile Phe Gln Val Ser Arg Asn Gln Ala Ile Lys
 300
 GAT GTT AAA GAA GGC GTG TTT GAG TTA AGC CGT TCT GCG GTA ATC TTT TAC CCG AAA GAA
 Asp Val Lys Glu Glu Gly Val Phe Glu Leu Ser Arg Ser Ala Val Ile Phe Tyr Pro Lys Glu
 360
 GGG AGT TTT GAC TGC GTC GCG CGC CCC TGG CTA ACA GAG GCT GGC AGC CGA TCA GCT CGT
 Gly Ser Phe Asp Cys Val Ala Arg Pro Trp Leu Thr Glu Ala Gly Ser Arg Ser Ala Arg
 420
 GGT ATC TGG GAA ATC GAA TTT AAC CAT AAA CTC CTG CCG TAC ATT TAC GGC CTG ACG AAC
 Gly Ile Trp Glu Ile Glu Phe Asn His Lys Leu Arg Tyr Ile Tyr Gly Leu Thr Asn
 480
 CAG TTC ACC ACC TAC TCG CTC CGC GAT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT TGT
 Gln Phe Thr Thr Tyr Ser Leu Arg Asp Cys Cys Gly Ser Leu Arg Asn Pro Arg Thr Ile Arg
 540
 CTT TAT GAA AGT CTT GCT CAA TTC AAA TCT TCA GGC TTA TGG GTT ACT ACT CAT GCT TGG
 Leu Tyr Glu Ser Leu Ala Gln Phe Lys Ser Ser Gly Leu Trp Val Thr Thr His Ala Trp
 600
 TTA AAT GAC CGT TTC CTT TTG CCG GAA TCC CAA CAG AAG AAC TTT GCA GAG TTG AAA CGA
 Leu Asn Asp Arg Phe Leu Leu Pro Glu Ser Gln Gln Lys Asn Leu Ala Glu Leu Lys Arg
 660
 TCT TTC CTT GAT CCT GCT CTC AAG CAG ATA AAT GAG AAA ACA CCT TTA CTT GCT AAG TAT
 Ser Phe Leu Asp Pro Ala Leu Lys Gln Ile Asn Glu Lys Thr Pro Leu Leu Ala Lys Tyr
 720
 AGT ATT GAT GAT TCA GGA AAA TTT CTG TTC TCA ATA ATT GAT AAG CAA AAT CCC GTC TGA
 Ser Ile Asp Asp Ser Gly Lys Phe Leu Phe Ser Ile Ile Asp Lys Gln Asn Pro Val End

Fig. 5.14. Design and effect of the site-directed mutations in the replicon of pCU1 (see also Fig. 5.13). Asterisks indicate the site of the mutations

Mutation I (nucleotide position 602 in Fig. 4.5)

Wild-type DNA sequence	:	5'-CTC ATG GAT AAG TTG CTG AAC AAA ...
Polypeptide sequence	:	Met Asp Lys Leu Leu Asn Lys ...
		★
Oligonucleotide sequence	:	3'-GAG TAC CTA ATC AAC GAC T-5'
Mutant DNA sequence	:	5'-CTC ATG GAT TAG TTG CTG AAC AAA ...
Polypeptide sequence	:	Met Asp END

Mutation II (nucleotide position 1116 in Fig. 4.5)

Wild-type DNA sequence	:	5'-TCA GGC TTA TGG GTT ACT ACT ...
Polypeptide sequence:		... Ser Gly Leu Trp Val Thr Thr...
		★
Oligonucleotide sequence	:	3'- GT CCG AAT ATC CAA TGA TG-5'
Mutant DNA sequence	:	5'-TCA GGC TTA TAG GTT ACT ACT ...
Polypeptide sequence	:	... Ser Gly Leu END

Fig. 5.15. Polypeptides synthesized *in vitro* from the DNA preparations of pCU714, pCU717 and pCU718. The big arrow indicates the MW 27,000 polypeptide encoded by the replicon of pCU1 and the protein encoded by the Km^r gene (see also Figs. 5.10-5.12). Note that in the case of pCU714 these two polypeptides co-migrate. In the case of pCU717, a truncated polypeptide of the MW 27,000 protein of the pCU1 replicon is synthesized (indicated by the small arrow). pCU718 does not direct synthesis of this polypeptide. For further details see Sections 5.2.10 and 5.3.2). Numbers on the left indicate MW ($\times 10^3$) of the marker proteins used.

PCU714
PCU717
PCU718

43

29

18

14

C2107 even at 30°C. The ability of pCU717 and pCU718 to transform CJ261 (or SR1672) could not be tested because both the two plasmids and the *E. coli* strains specify Km^r. The ability of pCU717 and pCU718 to be maintained in CC118 and Q125 is inferred as the ability of the mutations to be suppressed when the *E. coli polA* gene is provided.

Results shown in Fig. 5.15 demonstrate that mutation I abolished expression of ORF239 and that mutation II resulted in a truncation of the polypeptide of ORF239. This implies that the protein product of ORF239 is involved in the *polA*-independent replication of the pCU1 replicon.

In order to detect whether these mutations were suppressible, i.e., whether restoration of synthesis of the ORF239 protein would restore the *polA*-independent mode of plasmid replication, *E. coli* strain VNI101 was constructed. Briefly, the Cm^r gene was at first transduced from GE1730 into MBM7014 to yield VNI100 (frequency 1.2×10^{-5}). Then, the *supF* gene was transduced from VNI100 into SR1672 to yield VNI101 (frequency 3×10^{-7}). Transductants at this stage were selected for Cm^r phenotype and were screened for their ability to propagate λ ::TnphoA. λ ::TnphoA carries amber mutations in the essential genes of the lytic pathway, P and O genes and hence, can only be propagated in an *E. coli* host carrying amber suppressor mutation(s). Thus, VNI101 is a strain that contains a deletion of the *polA* gene and also carries a *supF* gene. It was found that neither pCU719 nor pCU720 could transform VNI101 (Table 5.7).

5.2.11. A plasmid containing the 2 kb region as the only replicon does not provide DNA repair functions

E. coli strains mutant in the *polA* gene are known to be UV-sensitive (see Sharma and Smith, 1987). In order to test whether the 2 kb replicon provided a functional analog

Table 5.7. Transformation of the mutant plasmids carrying base-substitutions in ORF239 (Section 5.2.10) into the *E. coli polA* mutant strains

Test plasmid	Frequency ^a of transformation of				
	CM4722	CJ261	CJ261 (pCU1)	CJ225	VNI101
pCU832	4.0×10^{-5}	9.0×10^{-4}	NT ^b	2.0×10^{-5}	NT
pCU714	5.5×10^{-5}	NT	NT	NT	NT
pCU717	2.5×10^{-5}	NT	NT	NT	NT
pCU718	1.5×10^{-5}	NT	NT	NT	NT
pCU45	7.0×10^{-5}	8.0×10^{-5}	$<10^{-9}$	4×10^{-4}	NT
pCU719	2.0×10^{-5}	$<10^{-9}$	$<10^{-9}$	1.5×10^{-5}	$<10^{-9}$
pCU720	2×10^{-5}	$<10^{-9}$	$<10^{-9}$	1.5×10^{-5}	$<10^{-9}$
pBR329	5.0×10^{-6}	$<10^{-9}$	$<10^{-9}$	8.0×10^{-6}	$<10^{-9}$

^aFrequency of transformants obtained/0.7 ug of plasmid DNA/ 10^7 cells is shown.

^bNT: Not tested.

of *E. coli* DNA polymerase I, the UV sensitivity of an *E. coli* strain carrying pCU832 was tested. Fig. 5.16 shows the survival curves of RR1, HB101, SR1784, SR1672 and SR1672(pCU832) following exposure to different doses of UV light. Plasmid pCU832 does not enhance the survival rate of SR1672 following exposure to the UV light.

5.2.12. A role for the 'GATC' sequence(s) present within the 2 kb region in plasmid maintenance

The DNA region between nucleotide co-ordinates 947-1315, that was previously inferred (Section 5.2.7) to contain the *cis*-acting region(s) contains three 'GATC' sequences at positions 1069-1072, 1194-1197 and 1205-1208 (Fig. 4.5) respectively. Potentially, these sites are sensitive to methylation by the *E. coli* Dam methylase at the N⁶ position of the adenine residue (Geier and Modrich, 1979). The restriction enzymes *Mbo*I and *Dpn*I are known to discriminate between methylated and non-methylated DNAs in a reciprocal way (Gelinas *et al.*, 1977; Lacks and Greenberg, 1975). *Dpn*I recognizes only the methylated 'GATC' tetranucleotide and cleaves between the guanine and adenine residues. Non-methylated 'GATC' is sensitive to *Mbo*I with the same endproducts as *Dpn*I cleavage on methylated DNA. pCU714 DNA isolated from HB101 (*dam*⁺) was found to be sensitive to *Dpn*I (Fig. 5.17). This DNA was used to transform *E. coli* GM48 (a *dam*⁻, *dcm*⁻ strain). Plasmid DNA isolated from GM48 (pCU714) was now resistant to *Dpn*I but sensitive to *Mbo*I (Fig. 5.17). Thus, it was clear that "methylated" and "non-methylated" pCU714 plasmid DNA preparations can be obtained by growing it in the appropriate *E. coli* backgrounds. Otherwise isogenic *dam*⁺ and *dam*⁻ strains of *E. coli* (strains 40 and BU1416 respectively) were then transformed with "methylated" and "non-methylated" pCU714 DNA preparations.

Fig. 5.16. UV-sensitivity curves for *E. coli* RR1 (○), HB101 (◻), SR1758 (⊙), SR1672 (◻) and SR1672(pCU832) (Δ).

Fig. 5.17. Agarose gel electrophoresis of plasmid DNA isolated from HB101(pCU714), lanes b, d and f and GM48(pCU714), lanes c, e and g. Plasmid DNA was restricted with enzymes *Bam*HI (lanes b and c), *Mbo*I (lanes d and e) and *Dpn*I (lanes f and g). Lane a contained 1 kb DNA MW standards.

The results shown in Table 5.8 indicate that the methylated plasmid DNA enhanced its maintenance ability.

5.2.13. Requirement of the *E. coli dnaA* gene for the maintenance of the pCU1 replicon

pCU1 was transferred from *E. coli* C600 to a Rif^r derivative of *E. coli* E177 (*dnaA* *ts*) at 30°C by bacterial conjugation. The transfer frequency was 10⁻². None of the transconjugants (>100) tested by replica plating grew at 42°C both on selective (for pCU1) and non-selective LA. At the same time, pCU1 was transferred from C600 to a Nal^r derivative of C600 (C600N) at a frequency of 10⁻¹ and was stably maintained at 42°C.

pCU1 was transferred from C600 to AQ3553 (*dnaA* *ts*) at 30°C at a frequency of 2 x 10⁻¹. While the transconjugants were viable at 42°C on LA, none of them (<1% survival) grew on LA containing Ap or Sp (pCU1-encoded antibiotic markers). pCU1 was transferred to AQ3563 (*dnaA::Tn10*) at a considerably lower frequency (2.2 x 10⁻⁴) than to AQ3553.

A non-self-transmissible but mobilizable deletion derivative of pCU1, pCU33 (see Fig. 3.4) was mobilized at a frequency thousand-fold lower to AQ3563 (frequency: 10⁻⁵) than to AQ3553 (frequency: 10⁻²) by pCU101. pCU101 was transferred at approximately equal frequencies to both AQ3553 and AQ3563 (10⁻²). The AQ3553(pCU33) transconjugants obtained at 30°C lost the plasmid at 42°C (as inferred from the loss of the plasmid-encoded antibiotic marker). As a negative control, it was shown that the plasmid F'Cm was transferred to AQ3553 at 30°C (frequency 10⁻¹). The transconjugants lost the Cm^r marker at 42°C. F'Cm-carrying transconjugants were not recovered in AQ3563 (<10⁻⁹).

The above results demonstrate that the *E. coli dnaA* gene is required for the maintenance of pCU1 and also for its deletion derivative pCU33. The *dnaA* gene has been

Table 5.8. Transformation of pCU714 isolated from the wild-type and *E. coli dam* strains (HB101 and GM48 respectively) into isogenic wild-type and *dam* strains of *E. coli*

Plasmid	Number of transformants per 100 ng of plasmid DNA/10 ⁷ cells	
	40	BU1416 (<i>dam</i>)

pCU714 (methylated)	480	61
pCU714 (non-methylated)	176	97

shown previously to be required for the maintenance of the F plasmid (F^{Cm}) but not for p15A (Kline *et al.*, 1986; pCU101 is a plasmid with a p15A replicon, Thatte and Iyer, 1983).

5.2.14. The 2 kb region encodes functions for the expression of plasmid incompatibility

Although previous experiments had established that the 2 kb *Pvu*II DNA region contained the minimal replicon of pCU1, it was not known whether this region also specified the plasmid's incompatibility properties. This was first established using plasmid pCU832 and pCU714 following which the various deletion derivatives and the subclones were tested. The incompatibility experiments were done with plasmids carrying dissimilar antibiotic-resistance markers and are summarized in Table 5.9. It can be seen that pRI120 carrying nucleotides 1-1315 of the 2 kb region displayed N incompatibility to pCU714. This indicates that there is an IncN determinant within this DNA region.

pRI105 that contains nucleotides 1374-2053 of the 2 kb region also expressed incompatibility towards a 2 kb plasmid (pCU832). The major feature of the 2 kb derived DNA in pRI105 is the Group I iterons. Thus, a second region of N incompatibility may be ascribed to this region. Interestingly, pRI119, that contains nucleotide co-ordinates 1017-1593 of the 2 kb region also specified the IncN phenotype. This, taken with the results of pRI105 suggests that only six members (and possibly fewer) of the Group I iterons are sufficient to encode plasmid incompatibility.

The mutations introduced into ORF239 (Section 5.2.10) did not affect the expression of plasmid incompatibility to pCU1 (Table 5.9). Thus, the replication determinants of pCU1 could not be separated from the incompatibility determinants.

Table 5.9. Plasmid incompatibility tests

Test plasmid ^a	Expression of incompatibility in GS500 carrying			
	pCU1	pCU714	pCU832	pRI103
pCU1		+	+	
pCU45	+	+		
pCU714	+		+	+
pCU719	+			
pCU720	+			
pCU744			+	
pCU773				+ ^b
pRI104		+	+	
pRI105			+	
pRI114		-		
pRI119	+			
pRI120		+		
pRI123		-		
pRI125		-		
pRI128		+		
pRI133		+	+	
pRI145	+			
pBR322, pBR328, pBR329	-	-		

^aThe test plasmid was at first introduced into GS500 carrying any one of the following four plasmids: pCU1, pCU714, pCU832, pRI103. Transformants were selected on LA containing an antibiotic to which the test plasmid and not the resident plasmid specified resistance. pCU1 was introduced into the recipient strain by conjugation. At least 50 transformants/transconjugants were replicated on LA without antibiotics and grown at 37°C. They were then tested for the presence of the test plasmid and/or the resident plasmid by replicating on LA with the respective antibiotics and incubation at 37°C. + indicates that 100% of the transformants/transconjugants had lost the resident plasmid except in ^b, where 70% of the cells had lost the resident plasmid. - indicates that 100% of the transformants/transconjugants retained the resident plasmid.

5.3. DISCUSSION

5.3.1. *E. coli* *polA* dependent and *polA* independent replication

In this study it was demonstrated that pCU1, pCU45, pCU714, pCU832 and pRI103 (Figs. 5.1 and 5.4) could each be maintained in the *polA* (*ts*) strain C2107 at 42°C. Under similar conditions, the pBR replicon was efficiently lost from C2107 at 42°C. The *polA*-independence of pCU1 and derivatives containing its replicon (pCU45, pCU714, pCU832 and pRI103) in CJ261 (and/or SR1672) was also shown. These results prove conclusively that the *E. coli* *polA* gene is dispensable for the maintenance of the pCU1 replicon. Replicons derived from F, P1, RK2, RSF1010, etc., have also been shown to be *polA*-independent (Scott, 1984; Pinkney *et al.*, 1988; Haring and Scherzinger, 1989). In all these cases, at least one plasmid encoded replication protein with a role in plasmid replication has been discovered (Watson *et al.*, 1982; Shingler and Thomas, 1984; Scherzinger *et al.*, 1984; Chatteraj *et al.*, 1985a,b; see also Table 1.2). The ORFs within the pCU1 replicon that could encode a putative replication protein(s) comparable in size to the replication proteins encoded by the other plasmid replicons are ORF239, ORF203, ORF188 and ORF245 (Section 4.2.3). Of these, a role for ORF245 in the replication and maintenance of pCU1 replicon was excluded by the construction of pCU751. A role for ORF203 and ORF188 may also be excluded because proteins of MW predicted by these ORFs were not detected in either minicells (Fig. 5.10) or in *in vitro* protein synthesis assays (Figs. 5.11. and 5.12). One or both of these assays had a resolving power that could identify such putative polypeptides if they had been synthesized in detectable amounts. Also, canonical ribosome binding sites for these ORFs could not be predicted from the DNA sequence (Section 4.2.3). The two independent polypeptide chain terminating point mutations that map within

ORF239 render the plasmid *polA*-dependent. Both these mutations abolished synthesis of the MW 27,000 protein (inferred to have been encoded by ORF239; Fig. 5.15). From these data it can be concluded that the DNA sequences of ORF239 contribute to the *polA*-independence function(s) of the pCU1 replicon. It is unlikely however, that ORF239 directly substitutes for the *polA* function. This view is supported by the results presented in Section 5.2.12, viz., a plasmid containing the pCU1 replicon (pCU832) did not reverse the hypersensitivity of a *polA* null mutant *E. coli* strain to UV light. MacPhee (1974) and Lehrbach *et al.* (1977) have reported that DNA polymerase activity was encoded by the other IncN plasmids R205 and pMG2 respectively. MacPhee's study was based on the demonstration that R205 (formerly called R-Utrecht) conferred UV-resistance to a *polA* strain of *S. typhimurium*. He inferred that a plasmid-encoded DNA polymerase participated in the repair of single-strand gaps caused by UV exposure. Lehrbach *et al.* showed that certain mutants of the plasmid pMG2 did not 'protect' the wild type and the *polA* mutant strain of *P. aeruginosa* from UV-induced lethality. Further, cell extracts of *P. aeruginosa* harboring pMG2 was shown to express a significantly higher DNA polymerase activity *in vitro*. Contradictory results were however, reported by Kronish and Walker (1979). In their study, Kronish and Walker did not find elevated DNA polymerase activity in extracts from *S. typhimurium* strains carrying R205 and pKM101 respectively. The results described in Section 5.2.11 support the conclusions of Kronish and Walker suggesting that the *polA*-independence of the pCU1 replicon is unlikely to have a basis in the synthesis of a plasmid replicon-specified *polA* analog.

The observations that both of the polypeptide chain-terminating base substitutions in ORF239 did affect a specific polypeptide (Fig. 5.13) and that they also affected the

ability of the plasmid to replicate in the *polA* mutant strains of *E. coli* support the notion that the ORF239 polypeptide is involved in the *polA*-independent mode of plasmid replication. However, a further expectation would be that if the effect of the polypeptide chain-terminating mutations were suppressed, the plasmid should now be able to replicate in the *polA* mutant. In a strain constructed to carry *supF* and the *polA* mutation, (and therefore presumed to be synthesizing ORF239-like polypeptides) the mutant plasmids still failed to replicate. Thus, both mutations, one at the N-terminal end of the polypeptide and another at its C-terminal half destroyed the plasmid's ability to be *polA*-independent and apparently did so in an irreversible way. However, it is not known at present whether an ORF239-like polypeptide is synthesized in VNI101. Perhaps the two mutations were introduced fortuitously into critical domains of the polypeptide. Further, it should be noted that the wild type amino acids at the locations of mutations I and II are lysine and tryptophan respectively (Figs. 5.13 and 5.14). The *supF* gene in VNI101 would be expected to substitute tyrosine for these amino acids (Eggertson and Soll, 1988). These results now emphasize a need for detailed *in vivo* and *in vitro* analysis of the ORF239 DNA sequence and its protein product using additional mutations directed to different regions of the polypeptide. It is nevertheless clear that in the absence of this *polA*-independent mode, the plasmid replicon is viable, utilizing the *polA* dependent mode. The interplay between these two replication modes and the definition of the circumstances when each or both are used are fascinating questions for the future.

5.3.2. The *trans*-acting region(s) of the pCU1 replicon

As described in Section 5.2.8, ORF46 must encode at least one *trans*-acting factor essential for pCU1 DNA replication. The replication proteins of other well characterized

replicons are larger compared to the predicted MW of ORF46 (MW 5500; Table 1.2). Regulatory roles have been ascribed to the smaller proteins specified by the other plasmid replicon systems (Molin *et al.*, 1981; Cesareni *et al.*, 1982; Thomas, 1988). It is not known now whether the product of ORF46 acts independently on the origin of replication of the 2 kb region or whether it interacts with another genetic product encoded from within nucleotides 1-1315 of the 2 kb region (e.g. the product of ORF239). That the polypeptide chain-terminating mutations within ORF239 abolished the *polA*-independence of the wild type 2 kb region showed that ORF239 specifies a polypeptide that is utilized by the pCU1 replicon for maintenance in wild-type *E. coli*. In such a case, ORF46 and ORF239 may act co-operatively in pCU1 DNA replication. The ability to rescue pRI104 *in trans* by pCU714 (Section 5.2.7) indicates that the ORF239 polypeptide can act *in trans*.

A role for the ORF239 protein in replication was also indicated in Section 5.2.10. The two mutations in ORF239 abolish expression of the MW 27,000 protein. In the case where mutation II was introduced, a truncated polypeptide (MW 12,000) was observed (Fig. 5. 15). However, the predicted MW of this truncated version of ORF239 was 20,000. Similar observations of apparent differences between the expected and observed mobilities of the replication protein have also been recorded by Perez-Casal *et al.* (1989). In such cases, the differences were attributed to the nature of the protein product.

The deletion of the Group I iteron family led to an increase in the plasmid copy number (plasmid pCU751). This indicates that (a) a positively acting factor(s) is involved in the replication of the pCU1 replicon and (b) the Group I DNA sequences serve to control the expression/action of such a positively acting factor(s). Interestingly, a 19 bp DNA sequence present partially within the left member of the Group II iteron family and partially outside it is represented straddling each member of the Group I iteron in an inverted

fashion (Kozlowski *et al.*, 1987; Section 4.2.3). This may lead to intrastrand hybridization and hence, formation of a hairpin loop structure. Three possibilities with regard to the expression of ORF46 and/or ORF239 could then be hypothesized: (a) the formation of the secondary structure described above may itself limit access of the regulatory sequences of ORF46 and/or ORF239 to the RNA polymerase. This mode of regulation is at the transcriptional level, (b) the transcriptional start site of ORF46 and/or ORF239 is to the left of the 19 bp DNA sequence mentioned above. This implies that the 5' domain of the mRNA synthesized from the ORF46 and/or ORF239 unit(s) could be titrated by the Group I iterons because of sequence homology. This mode of regulation is at the post-transcriptional level and (c) the polypeptide product(s) of ORF46 and/or ORF239 may bind to the Group I iteron and may thus be titrated. Examples of the titration of the *trans*-acting stimulatory factor of DNA replication by direct iteron families as in hypothesis (c) has been recorded in plasmids P1 (Chattoraj *et al.*, 1984), Rts1 (Kamio *et al.*, 1988) and R1162 (Lin and Meyer, 1986). While pCU751 showed a copy mutant phenotype, pCU752 did not (Fig. 5.9). Thus, of the thirteen members of the Group I iterons, six (and possibly fewer) are sufficient to control the copy number of the pCU1 replicon. The results presented in Table 5.5 support this conclusion. However, a strict correlation between the yield of plasmid DNA and the degree of antibiotic-resistance was not established. The mechanisms of plasmid replication and of antibiotic-resistance (Tc^r) are different and therefore, would be expected to yield different estimates of plasmid copy number.

While pCU751 exhibited a higher copy number than that of a 2 kb replicon (pCU832 and pCU714), the copy number of pCU752 was similar to that of the 2 kb replicons (Fig. 5.9). In addition to the presence of six members of the Group I iterons in

pCU752, it also carries ORF68 (Fig. 5.4). Therefore, it is possible that ORF68 may also be involved in plasmid copy number control.

The promoter of the gene encoding the replication protein in plasmid mini-Rts1 has been identified to include two inverted repeats of 8 bp and 6 bp respectively. The 8 bp repeat shows homology to a direct iteron family (three 21 bp repeats) established from the DNA sequence (called the *incII* repeats, Kamio *et al.*, 1984). The *incII* iteron is located upstream of the structural gene for replication protein in the mini-Rts1 map (Itoh *et al.*, 1987). A deletion involving these repeats has been shown to abolish origin functions. An absolute requirement of the direct iteron in front of the structural gene of replication for plasmid origin function has also been reported in pSC101 (Vocke and Bastia, 1983) and F (Disque-Kochem *et al.*, 1986). A 19 bp direct iteron family called *incE* region in plasmid pColV-K30 was postulated to form stem-loop structure involving the DNA sequences downstream of the *repI* gene (the structural gene encoding the replication protein) and the 18 bp direct iteron region represented within the putative promoter region of *repI* in tandem and at the C-terminal end of the *repI* gene (Perez-Casal *et al.*, 1989). Thus, the organization of the structural gene(s) for the replication protein(s) of several plasmids follows a general pattern of being preceded by sets of directly repeated DNA sequences that usually encompass the promoter of the *rep* gene. It is thus quite plausible that the Group II iterons precede the *rep* gene(s) of pCU1 and that they also include the promoter for these gene(s).

5.3.3. The *cis*-acting region of the pCU1 replicon

DNA sequences including nucleotide co-ordinates 947-1315 (Fig. 5.4) were inferred to contain the *cis*-acting function(s) for replication of the 2 kb region. Unlike the minimal

cis-acting regions of plasmids F (Kline, 1988), P1 (Abeles *et al.*, 1984), RK2 (Thomas and Helinski, 1989), R6K (Filutowicz *et al.*, 1985a), RSF1010 (Haring and Scherzinger, 1989) and R1162 (Meyer *et al.*, 1985), directly-iterated DNA sequences are absent in the *cis*-acting region(s) of the pCU1 replicon. Indeed, all of the Group I iterons are dispensable for plasmid replication (plasmid pCU751, Section 5.2.4). This is one of the unique features of the pCU1 minireplicon. The *cis*-acting region of pCU1 comprises three 'GATC' tetranucleotide sequences and one poor putative *dnaA* box (nucleotide co-ordinates 975-983, Table 4.2). Thirteen 'GATC' sequences and four *dnaA* boxes were identified within the minimal *cis*-acting region of the *E. coli* chromosome (*oriC*, 245 bp, Fuller *et al.*, 1984; Kornberg *et al.*, 1988). It has been postulated that the DnaA protein binds co-operatively to the *dnaA* boxes and causes local unwinding of the duplex DNA as an early step in the initiation of DNA replication of the *E. coli* chromosome (Bramhill and Kornberg, 1988). *dnaA* boxes in the replication origins of plasmids F, P1, pSC101, Rts1, pColV-K30 (Yarmolinsky and Sternberg, 1988; Perez-Casal *et al.*, 1989)) have been identified. The requirement of a functional *dnaA* gene has been demonstrated for pSC101 (Hasunuma and Sekiguchi, 1977), F and P1 (Hansen and Yarmolinsky, 1986; Kline *et al.*, 1986) and RK2 (Gaylo *et al.*, 1987). These observations and that DnaA is an initiator protein of replication (Kornberg *et al.*, 1988) has led to the speculation that several replicons adopt similar mechanisms in the initiation of DNA replication (Yeung and Dillon, 1988). In contrast, Itoh *et al.* (1987) and Itoh and Terawaki (1989) found that a deletion of the *dnaA* boxes of the mini-Rts1 plasmid did not abolish replication but reduced the copy number of the plasmid suggesting that a less efficient mode of replication when the *dnaA* boxes were deleted. A

reduction in plasmid copy number and stable inheritance was also observed when the *dnaA* box in the replicon of NR1 was deleted (Womble *et al.*, 1989).

It is noteworthy that fourteen putative DnaA-binding regions were recognized within a region of only 2 kb (Section 4.2.3, Table 4.2). A large number of *dnaA* boxes were also identified in the origin regions of *P. putida* (12 boxes) and *Bacillus subtilis* (14 boxes) (Fujita *et al.*, 1989). Whether any or all of the *dnaA* boxes in the pCU1 replicon is recognized by the DnaA protein remains to be established. It is possible that the presence of a large number of *dnaA* boxes is an insurance against mutational perturbations in the system. However, deletion of boxes 8-14 (Table 4.2) and 1 in independent experiments did not abolish autonomous replication of the 2 kb region (plasmid constructs pCU751 and pCU773 respectively). Also, deletion of boxes 1-6, with the retention of 7-14 (in the plasmid construct pRI104, see Fig. 5.4) did not affect the *cis*-acting functions of the plasmid. Presumably, in such recombinant plasmids, the alternative *dnaA* boxes are still available and provide the requisite recognition signals. In this regard, the non-canonical nature of the *dnaA* boxes merits mention. Therefore, if alternative *dnaA* boxes are indeed utilized, a different mechanism of DnaA-*dnaA* box recognition and interaction is possible. Of the fourteen putative *dnaA* boxes recognized, six are present within the Group I iterons (Fig. 4.6). In the light of this observation, it could be suggested that the evolution of these boxes was fortuitous i.e., the presence of these *dnaA* boxes is part of the evolution of the Group I iterons (analogous to the tandem direct iterons of plasmids like F, P1, Rts1, RK2, RSF1010, etc.) and that this alone would explain the large number of the *dnaA* boxes in the pCU1 replicon.

The presence of a large number of *dnaA* boxes may also explain the ability of pCU1 and pCU33 to be maintained in *E. coli* AQ3563, a *dnaA::Tn10* strain (Kline *et al.*, 1986). The site of Tn10 insertion in the *dnaA* gene of AQ3563 is currently not known. It is possible that in AQ3563 the *dnaA* gene is partially expressed and whatever DnaA protein is available (either reduced amounts or a truncated product) is sufficient to maintain the pCU1 replicon. It must be mentioned however, that Kline *et al.* (1986) reported that DnaA protein could not be isolated from AQ3563 by the standard biochemical techniques.

It is not concluded that the replication function(s) for pCU1 and pCU33 is provided by pKN500 (R1 replicon) in AQ3563 because then it would be expected that pCU1 and pCU33 would be maintained in AQ3553 also at 42°C. The utilization of a plasmid-determined genetic function(s) for the maintenance of another heterologous plasmid has been reported in the literature (Tait *et al.*, 1983). In this case, (plasmids pSa and R6K) homologous DNA sequences were observed within the origin regions of the two plasmids in question. No striking similarities in the DNA sequences of the replicons of R1 and pCU1 are however, noticed.

Methylation of the 'GATC' sequences within the *oriC* of the *E. coli* chromosome and the replicon of pBR322 have been observed (Messer and Noyer-Weidner, 1988; Russell and Zinder, 1987; Polisky, 1988). It has been suggested that such a methylation regulates the timing and initiation of DNA replication (Smith *et al.*, 1985). Messer and Noyer-Weidner (1988) noted that the adenine methylation of the 'GATC' sequences may provide signals for attachment of the DNA to the bacterial membrane. Bacterial membrane associated DNA replication has been demonstrated for plasmid RK2 (Firshein *et al.*, 1982). Membrane association may also provide support for the partitioning of the products of replication (Austin, 1988). Table 5.8 shows that while "non-methylated" pCU714 DNA

transformed the *dam*⁺ strain less efficiently than the "methylated" pCU714 DNA, the "non-methylated" pCU714 DNA transformed the *dam*⁻ strain more efficiently (50%) than the "methylated" pCU714 DNA. Russell and Zinder (1987) suggested that it was "hemi-methylation" that led to reduced maintenance ability of the plasmid pBR322. According to them, when the non-methylated DNA is transformed into a *dam*⁺ strain there is accumulation of the hemi-methylated form of DNA after one round of replication. Similarly, when the methylated DNA is transformed into a *dam*⁻ strain, there is accumulation of hemi-methylated DNA following one cycle of DNA replication. The signals for DNA replication of methylated and non-methylated DNAs may be independent of each other and hemi-methylation may simply serve as a blocking step for either modes of DNA replication. The possible existence of a methylation-independent DNA replication process is supported by the fact that *E. coli* strains with mutants of the *dam* gene could be isolated (both insertional and deletion mutants of the *dam* gene are available, Bakker and Smith, 1989; Marinus and Norris, 1973). Abeles and Austin (1987; 1988) showed that *dam* methylation was essential for DNA replication of P1 miniplasmid and not for miniF plasmid. The pCU1 replicon as represented by pCU714 showed a methylation-dependent enhancement of plasmid maintenance (Table 5.8). Therefore, the methylation of the 'GATC' sequences of the pCU1 replicon may not be involved in plasmid replication *per se*, unlike the P1 system, but may be involved in regulating the initiation of plasmid DNA replication as is the case in *E. coli* *oriC*.

It can be noticed from Table 5.6 that only a fraction of C2107 carrying pRI104 or pRI129 was rescued by pCU714 at 42°C. This may be attributed to the expression of incompatibility of these plasmids to pCU714. As was described in Section 5.2.14, the DNA region included in both pRI104 and pRI129 contains N incompatibility determinants.

Therefore, when they express incompatibility towards pCU714, a loss of this plasmid would be predicted. This means that the essential *trans*-acting function(s) required for the maintenance of pRI104 and pRI129 would be lost. This, in turn, would lead to a loss of pRI104 and pRI129 themselves at 42°C in C2107.

While pRI104 had the *cis*-acting DNA function(s) of the 2 kb region, neither pRI114 (and pRI115) nor pRI125 shared this property. This suggests that the separation of the *cis*-acting DNA at nucleotide position 1016-1017 affects the *cis*-acting ability of the region. In plasmids pRI114 (and pRI115) and pRI125 these DNA sequences are deleted in a complementary fashion. It is likely that the DNA sequences on both sides of nucleotide position 1016-1017 are required to be present *in cis* to each other for a functional *cis*-element.

From a comparison of the DNA sequences of various plasmid replicons, Filutowicz *et al.* (1985a) and Stalker and Helinski (1985) identified a pentanucleotide sequence 5'-ACAGG-3' to be present near the replication origin and a hexanucleotide sequence 5'-TGAGAG-3' within the direct iterons as probably being important features of these plasmid replicons. The ability of pCU751 to be maintained as a plasmid (Section 5.2.4) rules out that the above-mentioned hexanucleotide sequence is essential for plasmid replication in the pCU1 system. One of the two ACAGG (Section 4.2.3) is also deleted in pCU751. The second ACAGG is deleted in plasmid pCU773. It is not known whether both of these ACAGG sequences could be deleted from the pCU1 replicon without affecting its maintenance. However, it is clear that the *cis*-acting region of pCU1 does not exhibit this pentanucleotide sequence.

5.3.4. Plasmid incompatibility

Two domains of plasmid incompatibility were identified within the 2 kb region of pCU1. While one of these domains encompassed all members of the Group I iteron family (pRI105), the other excluded all of it (pRI120). Of the Group I iterons, just six members appear to be sufficient to encode plasmid incompatibility (plasmid pRI119; Section 5.2.14). This incompatibility determinant is designated *incA*. The role of tandem iterons in the expression of plasmid incompatibility has already been documented in plasmids F (Tolun and Helinski, 1981; Murotsu *et al.*, 1981), P1 (Abeles *et al.*, 1984; Chatteraj *et al.*, 1984), R1162 (Lin and Meyer, 1986; Lin *et al.*, 1987).

The basis of the expression of incompatibility by pRI120 is not clear. It could be mediated by the Group II iterons or by the cis-acting region of plasmid replication that would compete for an essential replication factor(s) (Novick, 1987). This second kind of pCU1 incompatibility determinant is designated *incB*.

5.3.5. Sequences determining plasmid host-range

It was established that plasmids containing the entire 2 kb PvuII region of pCU1 (the plasmid's replicon) had nearly the same broad-host-range as the parental plasmid (Chapter 3). An unexpected observation was that although pCU751 could transform *E. coli* C2107 at 30°C and be maintained at 42°C, it could not be transformed into the *E. coli polA* null mutant strains CJ261 and SR1672 (Table 5.4). This is not attributed as due to the total lack of the DNA polymerase I protein in these strains because transformants of pCU751 could not be recovered even in the respective wild type *E. coli* strains, CM4722 and SR1758. *E. coli* mutants that affect plasmid maintenance have been described by Biek and Cohen (1986; 1989). Notably, the DNA sequences of the 2 kb region that were deleted to

construct pCU751 include the Group I iterons. This implies that the nucleotides 1316-2053 (Fig. 4.5) have a role in determining the maintenance of the pCU1 replicon in these *E. coli* strains. The host-range of pCU751 in other Gram-negative purple bacteria has not been established. Schmidhauser and Helinski (1985) have described that additional DNA sequences of plasmid RK2 are required for the stability of the RK2 derived mini-plasmids in a host-specific fashion. These additional DNA sequences in some cases include directly-repeated segments of the DNA in the replicon of RK2.

5.3.6. A comparative discussion of the replicons of pCU1 and other plasmids

Based on the results described in Chapters 4 and 5, a functional organization of the replicon of pCU1 is proposed in Fig. 5.18. The origin(s) of plasmid replication is inferred to be contained in a 368 bp DNA segment. One poor putative *dnaA* box and three 'GATC' sequences were recognized within the origin region of the pCU1 replicon. These are features of the other replicons also (see Fig. 1.9). This suggests that the mode and regulation of plasmid DNA replication initiation is probably conserved in all these plasmid groups. Unlike in the origin region of several other plasmid replicons, e.g. P1, F, RK2 and RSF1010 (Stalker *et al.*, 1981a; Abeles *et al.*, 1984; Haring and Scherzinger, 1989), this region lacks directly-iterated sequences.

ORF239 was identified to provide at least one of the structural genes (*rep*) for plasmid replication. The region including ORF46 was determined to provide a *trans*-acting function for replication. The exact roles of the protein products of these regions are yet unknown. Notably, these regions are flanked by sets of direct-iterons and this is similar to the replicon organization of P1, F and ColV-K30 (Section 1.2). As in the case of pSa (Tait *et al.*, 1982), rescue of the origin-containing region requires the presence of an

Fig. 5.18. Functional organization of the replicon of pCU1. *ori* indicates the region inferred to contain the *cis*-acting region(s) for plasmid replication (Sections 5.2.7, 5.2.12 and 5.2.13). *cop* indicates the region that can function *in trans* to reduce copy number of a pCU1 replicon (Section 5.2.6). *rep* indicates the region inferred to contain the structural gene(s) for plasmid replication (Sections 5.2.4, 5.2.5, 5.2.8, 5.2.9 and 5.2.10). *incA* and *incB* are regions that encode plasmid incompatibility (Section 5.2.14). Only the *dnaA* box within the origin region is shown (by the arrow above the thick line). The location of the GATC sequences are represented by the closed circles. The other symbols are as in Fig. 4.6.

autonomously replicating unit of pCU1. However, unlike the situation in any other broad-host-range plasmid, the *ori* of the pCU1 replicon is contained entirely within a *rep* gene. It is interesting that all determinants for plasmid replication are contained within a small region of approximately 1 kb (Section 5.2.4).

The mechanism of determination of plasmid incompatibility by the pCU1 replicon does not appear to be different from replicons such as F, P1, Rts1 and R1162 (Novick, 1987). The tandem Group I iterons encode one kind of incompatibility and the origin containing region another kind. The Group I iterons also control plasmid copy number in the pCU1 system. Thus, the pCU1 copy number control mechanism may be analogous to that of F, P1 and Rts1 (Tsutsui *et al.*, 1983; Chatteraj *et al.*, 1984; Kamio *et al.*, 1984).

5.3.7. Further discussion

The purpose of this subsection is to highlight the following features of the pCU1 replicon that invite particular attention: (a) presence of the *cis*-acting region within a *rep* gene and the absence of direct iterons in the origin region for *E. coli* *polA*-independent replication and (b) the ability of the replicon to display both *E. coli* *polA*-independent and *polA*-dependent modes of plasmid maintenance.

The origins of replication of λ dv and pT181 have been mapped within the respective *rep* genes *O* and *repC* (Tsurimoto *et al.*, 1985; Khan *et al.*, 1982). Binding of the *trans*-active Rep proteins to the respective origins has also been demonstrated in such cases. It was suggested in Section 5.3.2 that the protein product of ORF239 probably represents the Rep protein of the pCU1 replicon. A feasible experimental approach to test this would be to clone the structural gene of ORF239 downstream of a strong promoter (e.g. *tac* promoter, de Boer *et al.*, 1983). This would facilitate the preparative isolation of the ORF239 protein.

By analogy to the Rep proteins of other plasmids, it can be predicted that the ORF239 protein will bind to the (i) inferred *ori* region (Section 5.3.3), (ii) Group I repeats (see Sections 5.2.6 and 5.3.2) and (iii) Group II repeats region (suggested to include the promoter of ORF239, Section 5.3.2). A demonstration that the Rep protein binds to the inferred *ori* region would provide confirmatory evidence that this is the *ori* region despite the lack of directly iterated DNA sequences within this segment. In this context, it is notable that the origin region of the staphylococcal plasmids pT181, pC221 and pUB112 also lacks direct iterons (Projan and Novick, 1988). However, they contain inverted iterons.

In addition to ORF239, ORF46 is also required for the replication of the pCU1 mini-replicon (Section 5.3.2). The introduction of polypeptide chain-terminating mutations in ORF46 (without introducing mutations in the overlapping ORF239) is expected to abolish synthesis of the 5500 MW protein encoded by the pCU1 replicon (Section 5.2.9). A role for the ORF46 protein in the *polA*-independent mode of plasmid maintenance can then be confirmed by examining the ability of the mutant derivatives to replicate in *E. coli polA*-strains. The preparative isolation of the ORF46 protein, as described above for ORF239 will then allow one to address the following questions: Does the ORF46 protein bind to the origin region? What is the nature of interaction between the proteins of ORF46 and ORF239?

The pCU1 replicon, uniquely, exhibits two modes of plasmid maintenance: *polA*-independent and *polA*-dependent. At present, it is not known whether the *cis*-acting region of the *polA*-independent mode is also functional in the *polA*-dependent mode. The several deletion derivatives obtained in this study provide a good starting point for the localization of the *polA*-dependent replicon. The approach would be to clone the pCU1 replicon sub-segments with a convenient antibiotic-resistance marker and examine its ability to replicate

in wild type and in *polA* *E. coli* strains with and without a 2 kb plasmid (e.g. pCU714). The ability of the derivative(s) to replicate in plasmidless wild type *E. coli* and not in *polA* *E. coli* would identify the *polA*-dependent replicon. The rescue experiments with pCU714 *in trans* would determine the *cis*-acting region(s) of such a replicon and also whether any *trans*-acting plasmid function(s) is involved in the *polA*-dependent mode of plasmid maintenance.

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