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**ISOLATION AND CHARACTERIZATION OF
MUTATIONS AFFECTING COPY NUMBER
CONTROL OF THE pCU1 REPLICON**

by

Ajoo Monica Narang

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

**Department of Biology
Carleton University
Ottawa, Ontario
1990**

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

**Isolation and Characterization of
Mutations Affecting Copy Number
Control of the pCU1 Replicon**

submitted by Ajoo Monica Narang, B. Sc.,
in partial fulfillment of the requirements for the
degree of Master of Science.

Chairman, Department of Biology

Supervisor

Carleton University

ABSTRACT

Two classes of spontaneous copy mutants of a mini-pCU1 plasmid were isolated. In both, increased copy number was observed following the isolation of their respective replicons. The first class of mutants, selected for increased ampicillin resistance (Ap^r), expressed a moderate increase in copy number. These mutants had base substitutions in open reading frames (ORF₂₃₉ and ORF₂₃₉-ORF₆₈ overlap region respectively). Neither mutation inactivated the Rep protein encoded by ORF₂₃₉. The second class of mutants were isolated by selection for Ap^r in the presence of a β -lactamase inhibitor. They expressed a level of Ap^r higher than the Class I mutants and had undergone deletion of sequences in a specific plasmid region (Group I repeat region). The copy number of the Class II mutants was reduced when the Group I repeat region was provided in *trans* supporting a role for these repeats in regulating copy number. A reduction was also observed with one of the Class I mutants that had an amino acid substitution in ORF₂₃₉. This substitution may have either resulted in the overproduction of the ORF₂₃₉ protein or in a reduction in its affinity for the Group I repeats. In the presence of ORF₆₈ in *trans*, a reduction in the copy number of both the wild-type replicon and the copy mutants was observed. These findings suggest that more than one element is responsible for copy number control of this replicon.

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LIST OF ABBREVIATIONS

Ap	Ampicillin
bp	Base pairs
Ci	Curie
CIAP	Calf intestinal alkaline phosphatase
cm	Centimetre
<i>cop</i>	Copy number genotype
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dTTP	Deoxythymidine triphosphate
ddH ₂ O	Distilled de-ionized water
ddNTP	Dideoxynucleoside triphosphate
DDT	Dithiothreitol
DNA	Deoxyribonucleic acid
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
g	Gram
³ H	Tritium
Inc	Incompatibility
IPTG	Isopropyl- β -D-thiogalactopyranoside
kb	Kilobase pairs
kDa	Kilodalton
Km	Kanamycin
LA	Luria-Bertani agar
LB	Luria-Bertani broth

M	Molar
mA	Milliamp
mg	Milligram
MIC	Minimum inhibitory concentration
ml	Millilitre
mM	Millimolar
mmol	Millimole
MW	Molecular weight
ng	Nanogram
-	Negative
ORF	Open reading frame
%	Percent
PEG	Polyethylene glycol
pmoles	Picomoles
+	Positive
<i>polA</i>	<i>E. coli</i> DNA polymerase I gene
^r/R	Resistance
<i>rep</i>	Replication gene
RF	Replicative form
RNA	Ribonucleic acid
RNase	Ribonuclease
Rom	RNA one inhibition modulator
Rop	Repressor of primer polypeptide
rpm	Revolutions per minute
SDS	Sodium dodecyl sulfate
Sm	Streptomycin
Sp	Spectinomycin

Tc	Tetracycline
Tris	Tris(hydroxymethyl)aminomethane
uCi	Microcurie
ug	Microgram
ul	Microlitre
UV	Ultraviolet
v/v	Volume per unit volume
w/v	Weight per unit volume
X-Gal	5-Bromo-4-chloro-3-indolyl-β-D-galactoside

1.0 INTRODUCTION

In addition to the genetic information stored on their chromosome, some bacteria contain accessory molecules of DNA, known as plasmids, that confer properties on their host that may prove beneficial for survival under certain conditions. Bacterial plasmids, being physically independent of the bacterial chromosome, replicate autonomously within the cell, often regulated only by plasmid-encoded functions. As a consequence of this and the technical ease by which these small extrachromosomal elements can be manipulated, plasmids have become useful as models for the study of regulation of DNA replication. Of particular relevance to this thesis is copy number control, the regulatory events that control the rate of initiation of replication resulting in the maintenance of a characteristic plasmid copy number.

From the literature on a few well-studied plasmids, it is apparent that a number of mechanisms have been evolved by plasmids to control their copy number. Such copy number control elements as RNA transcripts, small polypeptides and series of direct DNA repeats have been shown to have a role in determining plasmid copy number usually by limiting plasmid-encoded factors necessary for replication. Previous characterization of the replicon of the plasmid pCU1 has indicated that it possesses the potential for use of one or a combination of such elements for copy number control.

pCU1 is a 39 kb, naturally-occurring multicopy incompatibility group N plasmid that specifies ampicillin, streptomycin and spectinomycin resistance (Figure 1.0). The minimal replication region or replicon is contained within a 2 kb PvuII DNA fragment of pCU1 (Kozlowski *et al.*, 1987). The nucleotide sequence of the 2 kb replicon, summarized in Figure 1.0, revealed the presence of two groups of direct DNA repeats. The repeats, Group I and Group II, contain thirteen 37 bp tandem direct repeats and two and a partial third set of 30 bp

Figure 1.0 : Physical and genetic map of pCU1 and a miniplasmid derivative, pCU32, shown with the structural organization of its 2 kb replicon (thick solid bar). The rightward and leftward arrows immediately below the thick bar indicate potential reading frames inferred from the sequence. Closed and open arrowheads represent the Group I and Group II direct DNA repeats, respectively. Discontinuous lines indicate a 19 bp sequence involved in potential inverted repeat formation. Vertical arrows on the replicon represent 2 of a total of 14 dnaA boxes. Abbreviations: Sp^r, Sm^r and Ap^r, regions determining resistance to spectinomycin, streptomycin and ampicillin, respectively; ORF, open reading frame; A, AvaI; B, BglII; H, HpaI; Pv, PvuII; and S, SmaI.

repeats, respectively (Kozlowski *et al.*, 1987). Overlapping a member of the second group of repeats is a 19 bp sequence, also present in inverted orientation straddling the Group I repeats. Interaction of this sequence could result in the formation of hairpin loop-structures. Between these two repeats, there are a number of open reading frames of which only ORF₂₃₉ and ORF₄₆ could be shown to be expressed in an *in vitro* system (Krishnan, 1989). ORF₂₃₉ is believed to encode Rep, a protein required for replication. Krishnan (1989) excluded the functionality of ORF₂₀₃ and ORF₁₈₈, as putative proteins determined by them were not detected and their sequences were not preceded by ribosome-binding site sequences. However, Krishnan (1989) did leave open the possibility that ORF₆₈ and ORF₄₆ may have a role in plasmid replication. Deletion analysis showed that a cis-acting region, presumably containing the origin of vegetative plasmid replication, is entirely within the sequence determining the putative Rep protein (Krishnan, 1989). Similarly, it was shown that inactivation of the Rep protein renders the replicon *E. coli* *polA*-dependent (Krishnan, 1989). The present evidence suggests, therefore, that the replicon of pCU1 harbors a number of putative copy number control circuits, some of which may be unique to this plasmid, a representative of a large group of self-transmissible broad-host-range plasmids in gram negative bacteria.

To gain a better understanding of the role of these elements in copy number control, spontaneous replication mutants of pCU32 (a 6 kb ampicillin resistant miniplasmid derivative of pCU1) having altered copy number were isolated on the basis of increased antibiotic resistance (Figure 1.0). The analysis of these copy mutants assisted in localizing those structures in the replicon that directly or indirectly influence copy number. In addition, characterization of these copy mutations facilitated speculation, presented here, of the mechanism(s) of

regulation of replication expressed by the copy number control elements identified in this investigation.

1.1 MECHANISMS OF COPY NUMBER CONTROL: LITERATURE REVIEW

Under a defined set of conditions, plasmids replicate in a controlled way so as to maintain a certain characteristic number of copies per host cell indicating that plasmid replication is under the control of a specific regulatory mechanism. The isolation of copy number mutants of a variety of different plasmids suggests that plasmids play a role themselves in replication control (Iordanescu, 1983). Two different models have been proposed for copy number control. One model proposes a positively-acting element that regulates the initiation of replication while the second model postulates a negatively-acting element that functions to repress initiation (Jacob *et al.*, 1963; Pritchard *et al.*, 1969). For the replicons that have been studied in detail, copy number control has been attributed to three types of inhibitory element that function to repress initiation. They are: RNA transcripts, small polypeptides and series of direct DNA repeats.

1.1.1 RNA TRANSCRIPTS:

The utilization of RNA transcripts as replication inhibitors by a number of plasmids has been fairly well-characterized in the literature. This includes the *E. coli* plasmids ColE1 and CloDF13 (maintained at 15-20 copies/cell), the Inc FII group of plasmids of *E. coli* (1 copy/cell) and the *Staphylococcus aureus* plasmid pT181 (20-25 copies/cell) (Polisky, 1988; Womble and Rownd, 1988; Novick *et al.*, 1984). In all of these cases, the main inhibitor is a small RNA molecule (70-110 nucleotides long), with a high degree of secondary structure (often with one long stem and a 6-7 base single-stranded loop) which interacts with a long RNA transcript formed from the complementary strand from the same DNA region

(Nordstrom, 1985; Tomizawa, 1985; Wagner *et al.*, 1988). Initial interaction at the single-stranded loops is thought to result in the formation of a stable RNA duplex that changes the conformation of the longer transcript downstream. With respect to ColE1 and CloDF13, the processing of the long transcript ("preprimer" to primer) by the enzyme RNaseH is affected, due to the inability of the preprimer to hybridize stably to the DNA, thereby preventing the generation of a 3'-OH end which serves as the "primer" at which DNA synthesis is normally initiated by host DNA polymerase I (Davison, 1984). For pT181 and Inc FII plasmids, the consequence of RNA transcript interaction with the leading sequence of a messenger RNA is the inability to translate a Rep protein which is required and rate-limiting for replication of the plasmid due to the incorporation of the ribosomal binding site in the duplex or destabilization of the *rep* message, respectively (Nordstrom *et al.*, 1988; Riise and Molin, 1986; Novick *et al.*, 1984; Carleton *et al.*, 1984; Couturier *et al.*, 1988). In all cases, mutations that disrupt the secondary structures of the transcripts and so affect RNA-RNA interaction result in changes to plasmid copy number (Davison, 1984; Polisky *et al.*, 1990; Carleton *et al.*, 1984; Nordstrom *et al.*, 1988).

1.1.2 Small Polypeptides:

The Inc FII plasmids and ColE1 encode a second type of inhibitor, a small polypeptide that is a negative, trans-acting element. Inc FII plasmids contain the structural gene, *copB*, which codes for a small basic protein (Nordstrom and Nordstrom, 1985). The constitutively synthesized CopB protein, under normal conditions, represses transcription by binding to the DNA region that overlaps with the *rep* gene promoter (Banerjee *et al.*, 1990; Riise and Molin, 1986). ColE1 also encodes a small protein known as Rop (repressor of primer polypeptide) or Rom (RNA one inhibition modulator) (Bujard *et al.*, 1985; Brenner and Tomizawa,

1989). The *rom* gene, located downstream from the origin, encodes a small protein of 63 amino acid residues which enhances the stability and rate of pairing of the small, highly structured RNA molecule (inhibitor) to the long transcript (preprimer) formed from the complementary strand (Polisky *et al.*, 1985; Perelson and Brendel, 1989). As a result, the formation of the primer at which DNA synthesis is initiated, is inhibited.

A similar mechanism of control has also recently been reported for the *Bacillus* plasmid, pRBH1, a drug resistance plasmid found at a copy number of 42 copies/cell (Muller *et al.*, 1986). Initiation of plasmid replication has been proposed to be regulated by the interaction of a long primer transcript with a small RNA molecule transcribed from the complementary strand downstream of a Rep protein coding sequence (Ano *et al.*, 1986). In addition, homology between the plasmid-encoded Rep protein and the Rom protein of ColE1 has been observed indicating that the Rep protein may also act directly to enhance binding of the two complementary RNAs, thus inhibiting its own synthesis and ultimately replication (Muller *et al.*, 1986). Indeed, increases in copy number have been observed as a result of single base changes in the Rep protein coding sequence thereby implicating its role in regulation of replication (Muller *et al.*, 1986).

Small polypeptides have also been implicated as replication inhibitors in other plasmids. In the case of the broad-host-range plasmid RK2 (maintained at 4-7 copies/cell in *E. coli*), two proteins KorA (also referred to as TrfB) and KorB have been shown to negatively regulate the expression of TrfA, a protein required for replication (Thomas and Hussain, 1984; Thomas and Smith, 1987; Thomas and Helinski, 1989). Another broad-host-range plasmid, RSF1010 (10-12 copies/cell in *E. coli*), encodes a 7 kDa protein that has been shown to bind to the promoter that controls the expression of 2 *rep* genes including *repC*, a gene encoding a protein positively required for replication (Sholz *et al.*, 1989; Frey and Bagdasarian, 1989).

Similarly, footprinting experiments have confirmed that a small 5.1 kDa protein (RepA), encoded by the promiscuous streptococcal multicopy plasmid pLS1, limits synthesis of the initiator RepB protein by binding to its promoter (del Solar *et al.*, 1989).

1.1.3 Series of Direct DNA Repeats:

The third type of inhibitor of initiation of DNA replication is a series of direct DNA repeats. Direct repeats have been found to fulfill a variety of roles in those replicons that have been investigated thus far. For example, studies of low copy number (1-2 copies/cell) *E. coli* plasmids F, P1 and Rts1 indicate that the repeats directly regulate copy number by non-productive binding of plasmid-encoded Rep protein (Womble and Rownd, 1987; Terawaki *et al.*, 1990; Abeles *et al.*, 1989). In all three cases, the direct repeats (19-24 bp) are found as clusters flanking an open reading frame (ORF) encoding a replication protein (Tsutsui *et al.*, 1983; Pal *et al.*, 1986; Terawaki and Itoh, 1985). Deletion analysis has shown that one cluster of repeats is required for initiation of replication at the origin while the second set of repeats modulate copy number by titration of the positively required Rep protein (Tsutsui *et al.*, 1983; Pal *et al.*, 1986; Terawaki and Itoh, 1985). Similarly, single base pair changes made in the *rep* coding region of plasmid Rts1 resulted in an increased copy number due to the lowered affinity of the Rep protein for the repeats (Terawaki and Itoh, 1985). As well, the effect of increased copy number, as a result of providing excess plasmid F Rep protein in *trans*, could be counteracted by increasing the copies of the direct repeats (Rokeach *et al.*, 1985). There is also evidence from work on plasmid P1, that the direct DNA repeats may limit replication by disrupting origin function as a result of steric hindrance caused by the interaction of the direct repeat-Rep complex with the origin (Pal and Chatteraj, 1988; Abeles *et al.*, 1989). The direct repeats found in

these plasmids and R6K, an *E. coli* plasmid maintained at 15-20 copies/cell, are also thought to serve as part of the operator site whereby interaction of the Rep protein with the operator results in the autogeneous control of transcription of the *rep* gene (Rokeach *et al.*, 1985; Terawaki *et al.*, 1990; Abeles *et al.*, 1989; Kelley and Bastia, 1985).

A slightly more complex mechanism of control is evident for the gram negative broad-host-range plasmids, RK2 and RSF1010, present at 4-7 copies/cell and 10-12 copies/cell in *E. coli* respectively (Thomas and Hussain, 1984; Haring *et al.*, 1985). For instance, replication of RSF1010 requires three plasmid-encoded proteins that are clustered in one region of the plasmid, away from the origin of replication (Scherzinger *et al.*, 1984). However, only Rep protein (RepC) positively regulates replication (Haring *et al.*, 1985). Experiments have shown that purified RepC protein binds to RSF1010 DNA fragments containing the 20 bp direct repeats of the origin region (Haring *et al.*, 1985). Hence the direct repeats, in this instance, serve to direct initiation proteins to the replication origin.

Direct repeats are also found adjacent to the vegetative origin of RK2 (Thomas and Hussain, 1984). Deletions of one or both sets of direct repeats (*copA* and *copB*) results in increased copy number (Thomas *et al.*, 1984). It is postulated that these elements control the rate of replication by titrating TrfA, a protein required for replication (Rep), or by DNA folding caused by direct repeat-TrfA-origin interaction (Thomas and Smith, 1987; Thomas and Helinski, 1989). However, as previously mentioned, the overriding copy number control mechanism is known to be provided by two small plasmid-encoded proteins, KorA and KorB that inhibit transcription of the plasmid-encoded *trfA* gene. However, these proteins are not sufficient to compensate for the effect of loss of the direct repeats on copy number. Another gene in the *korA* operon, *incC*, is required (Thomas and Helinski, 1989). In the presence of a functional *korA-incC-korB* operon, the

deletion of *copA* and/or B has little effect on copy number (Thomas and Smith, 1989).

1.2 Host-Encoded Factors:

Although plasmid copy number is generally determined by plasmid-mediated regulatory mechanisms, a few host-encoded factors, recently identified, have been shown to directly affect copy number. For example, mutations in the *E. coli pcnB* locus result in the reduction of copy number of ColE1-like plasmids (Liu and Parkinson, 1989). Nucleotide sequencing has revealed that *pcnB* is the first gene in an operon and should encode a 47.3 kDa protein. From the amino acid sequence of the *pcnB* product, it is predicted that the protein is basic and so capable of binding to DNA or RNA (Liu and Parkinson, 1989). However, just how the *pcnB* product affects copy number remains to be determined. Conversely, mutations in the *E. coli recD* gene result in an increase in the copy number of several small plasmids (Seelke *et al.*, 1987). The *recD* locus, shown to affect exonuclease V activity and plasmid maintenance and recombination, allows plasmids to utilize the rolling circle mode of replication when mutated resulting in an increased frequency of linear plasmid multimer formation (Biek and Cohen, 1986; Seelke *et al.*, 1987). It is suggested that site-specific recombination can resolve these linear plasmid multimers into covalently closed circular plasmid DNA forms. As a result of the combination of this type of replication mode with an active site-specific recombination system, increased plasmid copy number is observed (Seelke *et al.*, 1987). Likewise, *Staphylococcus aureus* chromosomal mutations which reduce the copy number of pT181 and its derivatives have been isolated (Iordanescu and Bargonetti, 1989). It has been shown that these mutations, called *pcr* for plasmid copy number reduction, do not interfere with the synthesis of the Rep protein, RepC, that is necessary for replication (Iordanescu

and Bargonetti, 1989). However, there is some initial evidence suggesting that the *pcr* product may interact with the origin or RepC and so limit replication (Iordanescu and Bargonetti, 1989).

2.0 MATERIALS AND METHODS

2.1 Bacterial strains, Plasmids and Bacteriophages:

The bacterial strains, plasmids and bacteriophages used in this study can be found in Tables 2.0-2.2.

2.2 Growth Media:

Cells were routinely grown in modified L Broth or LB (5g NaCl, 5g yeast extract and 10g tryptone per litre) or on L Agar or LA plates (modified L broth with 1.6% w/v agar). SR1672 was grown in TY Broth or TYB (13g yeast extract and 5g tryptone per litre) or on TY Agar or TYA plates (TY broth with 1.6% w/v agar). M13 recombinants were isolated on H plates (8g NaCl, 10g tryptone and 12g agar per litre) using molten H top agar (8g NaCl, 10g tryptone and 8g agar per litre).

2.3 Antibiotics:

The following antibiotics were obtained commercially from the Sigma Chemical Company: sodium ampicillin, kanamycin monosulfate and tetracycline hydrochloride. The concentration of these antibiotics used normally for growth and selection was 30 μ g/ml, 50 μ g/ml and 20 μ g/ml respectively.

2.4 Enzymes:

Restriction endonucleases, T4 DNA ligase and 5x ligation buffer were obtained from New England Biolabs or Bethesda Research Laboratories. Calf intestinal alkaline phosphatase (CIAP) was obtained from Boehringer-Mannheim Canada Ltd.

Table 2.0 : Genotype of bacterial strains used.

Strain	Genotype/Phenotype	Source/Reference
DH5aF'	<u>endA1</u> <u>hsdR17</u> <u>SUP</u> E44 Δ <u>thi-1</u> <u>recA1</u> <u>gyrA</u> <u>relA1</u> $\emptyset$ 80 Δ <u>lacZ</u> M15 Δ (<u>lacZ</u> Y Δ - <u>argF</u>)U169	Liss, 1987
HB101	F' <u>hds</u> S20 <u>recA13</u> <u>ara-14</u> <u>proA2</u> <u>lacY1</u> <u>galK2</u> <u>rpsL20</u> <u>xy1-5</u> <u>mtl-1</u> λ	Bolivar, 1977
SR1672	Δ (<u>gal-bio</u>) <u>thi-1</u> <u>relA1</u> <u>spoT1</u> Δ <u>polA</u> Km ^R F ⁺	Sharma and Smith, 1987
SR1758	Δ (<u>gal-bio</u>) <u>thi-1</u> <u>relA1</u> <u>spoT1</u> F ⁺	Sharma and Smith, 1987

Table 2.1 : Description of plasmids used.

Plasmid	Antibiotic Marker ^a	PCU1 Replicon Coordinates ^b	Source/Reference
PBR322	Ap, Tc	-	Bolivar et al., 1977
PCU32	Ap	1-2053	Konarska-Kozłowska et al., 1983
PCU32COB3 ^c	Ap	1-2053	This study
PCU32COB75 ^c	Ap	1-2053	"
PCU32COB1 ^d	Ap	1-1448;1820-2053	"
PCU32COB1 ^d	Ap	NS ^e	"
PLG33 ^f	Km	-	Stoker et al., 1982
PMN321	Km	1-2053	This study
PMNCOB3 ⁹	Km	1-2053	"
PMNCOB75 ⁹	Km	1-2053	"
PMNCOB1 ⁹	Km	1-1448;1820-2053	"
PMNCOB1 ⁹	Km	NS ^e	"
PMNCOB1 ⁹	Km	1375-2053	Krishnan, 1989
PMNCOB1 ⁹	Ap	1-1016	"
PMNCOB1 ⁹	Ap, Tc	1017-1593	"
PMNCOB1 ⁹	Ap	1825-2053	"
PMNCOB1 ⁹	Ap, Km, Tc	-	D.R. Helinski
PMNCOB1 ⁹	Ap, Sm	-	R. Clowes

^aIndicates resistance to ampicillin (Ap), tetracycline (Tc), kanamycin (Km) and streptomycin (Sm).
^bSee Figure 1.0.

^cThese plasmids carry point mutations within the replicon.

^dThese plasmids carry deletions within the replicon.

^eReplicon has not been sequenced.

^fDerivatives harboring 2 kb wild-type or copy mutant replicon respectively.

Table 2.2 : List of Bacteriophages used.

Bacteriophage	pcu1 Replicon Coordinates ^a	Source/Reference
M13mp19	-	Yanisch-Perron et al., 1985
M13BOB1	1-2053	Krishnan, 1989
M13BOB2	1-2053	"
M13cop3-1 ^b	1-2053	This study
M13cop3-2 ^b	1-2053	"
M13cop75-1	1-2053	"
M13cop75-2	1-2053	"
M13copI-1	1-4998;1820-2053	"
M13copI-2	1-1448;1820-2053	"

^aSee Figure 1.0.

^b-1 and -2 indicate the orientation of the M13 strands i.e. that of M13BOB1 and M13BOB2 respectively.

2.5 Chemicals:

Clavulanic acid was obtained as a gift from Beecham Pharmaceuticals (Surrey, England). All other chemicals used were of reagent grade. Oligonucleotide primers used in this study were obtained as a gift from NRC (Ottawa, Canada).

2.6 Radiochemicals:

Deoxyadenosine 5'-[^{35}S] thiotriphosphate, triethylammonium salt (>1000 Ci/mmol) and methyl-[^3H] thymidine (5 Ci/mmol) were obtained from Amersham Canada Ltd.

2.7 Buffers and Solutions:

Plasmid DNA, single-stranded DNA and DNA fragments were resuspended in TE buffer (10 mM Tris-Cl pH 8.0 and 1 mM EDTA pH 8.0). DNA samples were prepared for agarose gel electrophoresis by adding stop solution (7M urea, 50% sucrose, 50 mM EDTA, 0.025% xylene cyanol and 0.025% bromophenol blue). Agarose gel electrophoresis was carried out using TAE buffer (40 mM Tris-Cl, 20 mM sodium acetate and 2 mM EDTA, pH 7.6) or TBE buffer (89 mM Tris-Cl, 89 mM boric acid and 2.5 mM EDTA, pH 8.2). All restriction digests were performed using the universal or TA buffer (33 mM Tris-acetate pH 7.9, 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM dithiothreitol and 100 g/ml gelatine).

2.8 Determination of Relative Antibiotic Resistance:

Various dilutions of overnight cultures grown in LB with the appropriate antibiotic depending on the plasmid used, were spread onto LA plates containing varying concentrations of antibiotic. Percent survival was determined by

calculating the ratio of the number of transformants at a particular concentration to the number of transformants at the "normal" concentration (30 $\mu\text{g/ml}$ for ampicillin). This value was multiplied by 100. In other cases, transformed cells growing at the normal concentration of antibiotic were replica-plated by toothpick onto LA plates of increasing antibiotic concentration. In either case, the concentration of antibiotic that prevented growth after overnight incubation at 37° C was taken to represent the minimal inhibitory concentration (MIC) of antibiotic.

2.9 Isolation of Mutants with Increased Plasmid Copy Number:

Cultures of HB101(pCU32) were grown, in 5 ml of LB containing 30 $\mu\text{g/ml}$ of ampicillin, overnight at 37° C with aeration. 0.1 ml of the overnight cultures were spread directly onto LA plates containing ampicillin at a concentration above the MIC (300-750 $\mu\text{g/ml}$). In some experiments, this medium was supplemented with 1 $\mu\text{g/ml}$ of clavulanic acid, a β -lactamase inhibitor that effectively reduced the MIC of ampicillin by about 50-fold so that lower concentrations of ampicillin (150 $\mu\text{g/ml}$) could be used for mutant isolation. The resulting resistant colonies were cultured in 5 ml of LB containing the elevated level of ampicillin and again spread onto LA plates containing various concentrations of ampicillin. Resistant colonies isolated in the presence of clavulanic acid were replica-plated by toothpick onto LA plates containing 1 $\mu\text{g/ml}$ of clavulanic acid and increasing levels of ampicillin. Cells with increased resistance were isolated as putative copy number mutants. Increased antibiotic resistance as a result of mutation to plasmid copy number control element(s) and not to mutations to the host was determined by isolating plasmid DNA from cells with increased resistance by rapid extraction and transformation of *E. coli* HB101 cells. Maintenance of increased antibiotic resistance in transformants was determined by replica-plating onto LA plates

containing increasing concentrations of ampicillin with or without 1 g/ml of clavulanic acid.

2.10 DETERMINATION OF COPY NUMBER

2.10.1 Modified Eckhardt Procedure (Projan *et al.*, 1983):

Overnight cultures grown in LB with the appropriate antibiotic depending on the plasmid used, were diluted 1:50 in fresh LB with antibiotic and grown to log phase at 37 C with aeration. Cell densities were monitored at 600 nm until all samples reached a standard density. An equal volume of cells (1.2 ml) from each sample was harvested by centrifugation in a 1.5 ml Eppendorf tube for 1 minute. Each cell pellet was resuspended in 100 μ l lysing buffer (200 mM Tris-Cl pH 8.0, 10 mM EDTA, 100 mM NaCl, 20% (w/v) sucrose, 25 g/ml lysozyme and 5 u/ml RNase). The suspension was incubated at 37 C for 30 minutes. Lysis was accomplished by the addition of 100 μ l of 2% SDS. The lysate was vortexed at top speed for 2 minutes, frozen and thawed twice and treated with 2 μ l of 1 mg/ml proteinase K for 30 minutes at 37 C. Following the addition of 50 μ l dye solution (50% glycerol, 0.02% xylene cyanol, 0.02% bromophenol blue), samples were electrophoresed on a vertical 1% agarose gel run in 1x TBE buffer at 2.5 volts/cm overnight. The gel was stained in 1 μ l/ml ethidium bromide for 30 minutes. After destaining in water for 90 minutes, the gel was photographed under UV illumination using Polaroid Type 55 positive/negative film. The relative quantities of plasmid and chromosomal DNA were determined from a densitometric scan of the film negative using the Hoeffer Scientific GS300 Transmittance/Reflectance Scanning Densitometer and were used to calculate copy number using the equation: $C_p = G_h \times R_p / G_p \times R_h$

where C_p represents the plasmid copy number per cell, G_h and G_p represent the chromosomal and plasmid DNA size, in bp respectively, and R_p and R_h represent the amounts of plasmid and chromosomal DNA in the gel, respectively.

2.10.2 [^3H]-Thymidine Incorporation (Taylor and Brose, 1988):

Cells, containing plasmids, were grown overnight in 1 ml of LB in the presence of 10 μCi of ^3H -thymidine at 37° C with aeration. The cells were harvested, in Eppendorf tubes, by centrifugation using a microfuge for 1 minute. The cell pellet was resuspended in 100 μl of solution A (25 mM Tris-Cl pH 8.0 and 50 mM glucose) and incubated on ice for 20 minutes. Lysis was accomplished by the addition of 200 μl of freshly prepared solution B (0.2 M NaOH and 1% SDS) and incubation on ice was continued for another 5 minutes. The lysate was kept on ice for another 60 minutes following the addition of 150 μl of 3M sodium acetate, pH 4.8. Precipitate was collected by centrifugation for 10 minutes. The supernatant was transferred to a fresh Eppendorf tube. The pellet of chromosomal DNA and cell debris was resuspended in 50 μl TE buffer. Plasmid DNA was precipitated from the supernatant by the addition of 1 ml of 95% absolute ethanol. Tubes were incubated on ice for 5 minutes. The precipitate was pelleted by centrifugation for 5 minutes. The supernatant was discarded and the precipitate was air dried. The precipitate was redissolved in 50 μl TE. To both chromosomal and plasmid DNA fractions, 1 ml of concentrated perchloric acid was added and samples were incubated at 85° C for 30 minutes. The samples were mixed with 10 ml of aquasol and the radioactivity of each fraction was measured using a liquid scintillation counter. Plasmid copy number was calculated using the equation described in Section 2.10.1 where C_p is the plasmid copy number per cell, G_h and G_p represent the size, in bp, of the host chromosome and plasmid

respectively, and R_p and R_h represent the radioactivity, in cpm, of the plasmid and chromosomal fraction respectively.

2.11 Incompatibility:

A plasmid altered in copy number was introduced by transformation into a recombination-deficient strain (Rec⁻ A⁻) harboring the wild-type miniplasmid derivative (pMN32), selecting for Ap^r (marker of incoming plasmid). To release selection, approximately 100 colonies were replica-plated by toothpick onto LA plates and grown overnight at 37° C. Retention of the resident and/or incoming plasmid in these cells was determined by replica-plating onto media selecting for either plasmid.

2.12 Shotgun Cloning: Construction of Copy Mutant Km^R Recombinants:

DNA of the copy mutants of pCU32 and of pLG338 (1 µg of CsCl purified DNA each) was digested separately in a 20 µl volume using 5 units of PvuII in the presence of TA buffer at 37° C for 90 minutes. The digested DNA was ethanol precipitated in the presence of 10 mM ammonium acetate for 1 hour on ice and resuspended in 10 µl of TE buffer. An equal volume of PvuII digested pCU32 or pCU32 copy mutant DNA to pLG338 PvuII digested DNA was combined in a 10 µl volume in the presence of 1 unit of T4 DNA ligase. The ligation mixtures were incubated overnight at 14° C. Half of the ligation mix was used per transformation, selecting for kanamycin resistant bacteria. Transformants were replica-plated onto kanamycin, ampicillin and tetracycline selective media. Plasmid DNA from ampicillin and tetracycline sensitive and kanamycin resistant recombinants was isolated by rapid extraction. Copy number of these miniplasmid derivatives was determined according to Section 2.10.2.

2.13 Complementation Analysis:

Various cloned derivatives of the 2 kb replicon (see Figure 2.0) were introduced by transformation into a recombination-deficient strain (Rec A⁻) harboring the kanamycin resistant test plasmids (wild-type or copy mutant replicons). Transformants were isolated on media containing antibiotics selecting for both the incoming and resident plasmids. Changes in resistance, conferred by the resident plasmid as a result of cohabitation, were determined by replica-plating cells onto LA plates containing increasing levels of kanamycin and the normal concentration of antibiotic selective for the deletion derivative. Cells that displayed a consistent change in resistance, conferred by the resident plasmid in the presence of a specific deletion derivative, were regrown to a standard density before plasmid DNA was isolated by rapid extraction. Samples were electrophoresed on a 0.8% agarose gel in 1x TBE buffer to correlate changes in resistance to plasmid DNA yield.

2.14 PolA Dependence or Independence:

Overnight cultures of SR1672 (*polA* null mutant) grown in TYB and kanamycin and its isogenic *polA*⁺ strain SR1758, grown in LB, were diluted 1:50 into 100 ml of TYB and LB respectively. The cells were grown at 37 C to early log phase. The cells were collected by centrifugation at 6000 rpm for 10 minutes at 4 C. Cell pellets of each strain were pooled and resuspended in 15 ml of cold 0.1M MgCl₂. Cells were reharvested by centrifugation as before. Each cell pellet was resuspended in 2 ml of cold 0.1M CaCl₂ and kept on ice for approximately 16 hours. In Eppendorf tubes, 0.2 ml of these competent cells were added to each 0.5 g DNA sample and incubated on ice for 30 minutes after gentle mixing. In addition to the test plasmids, pBR322, a *polA* dependent plasmid and pLG338, a *polA* independent plasmid, were used as controls. Tubes were heat shocked for 5

Figure 2.0 : Derivatives of pBR322 carrying different regions from the pCU1 2 kb miniplasmid replicon shown in relation to the physical map of the replicon.

minutes at 37° C. To each tube, 0.8 ml of TYB or LB was added according to the strain used, and the tubes were incubated at 37° C for 1 hour. The entire transformation mixture was plated on selective media. The lack of growth of transformed SR1672, after overnight incubation at 37° C, was taken to represent PolA dependence.

2.15 DNA SEQUENCING

2.15.1 Preparation of Vector DNA:

Approximately 500 ng of M13mp19 RF DNA was digested with 8 units of Sma I in the presence of TA buffer in a 20 μ l volume at 30° C for 60 minutes. The extent of RF DNA digestion was assessed by comparing the relative electrophoretic mobilities of digested and undigested vector on a 0.8% agarose gel run in 1x TBE buffer. The completely digested vector was purified by phenol extraction and ethanol precipitation. The digested vector precipitate was resuspended in TE buffer to a final concentration of 10 ng/ μ l. In a 40 μ l volume, 1 unit of CIAP was added to the dissolved vector in the presence of CIAP buffer (20 mM Tris pH 9.2, 0.2 mM EDTA). The reaction mixture was incubated at 45° C for 30 minutes. A further 1 unit of CIAP was added to the mixture which was re-incubated for another 30 minutes at 45° C. The reaction was stopped by phenol extraction and the CIAP-treated vector DNA was recovered by ethanol precipitation, resuspended in TE buffer to a final concentration of 10 ng/ μ l and stored at 4° C until further use.

2.15.2 Preparation of Insert DNA:

The 2 kb fragment of DNA to be sequenced was recovered using agarose gel electrophoresis and "GeneClean" as instructed by the manufacturer (Bio101

Inc., La Jolla, CA.) following a restriction digest of the appropriate plasmid using PvuII.

2.15.3 Ligation:

Ligations were carried out as indicated in section 2.16.5 with the ratio of insert to vector DNA as 5:1.

2.15.4 Transformation:

An overnight culture of DH5 α F', grown in LB, was diluted 1:20 into 40 ml of LB. The cells were grown at 37° C with aeration for 2 hours. Cells were harvested by centrifugation at 7000 rpm for 4 minutes. The cell pellet was resuspended in 20 ml of cold 50 mM CaCl₂ and incubated on ice for 20 minutes. The pellet was resuspended in 4 ml of cold 50 mM CaCl₂ and kept on ice for at least 2 hours before use. During this time, a fresh exponential phase culture of DH5 α F' was grown by adding a drop of overnight culture to 5 ml of LB.

In chilled Eppendorf tubes, 0.3 ml of competent DH5 α F' cells were added to each half of the ligation mix, and were incubated on ice for at least 45 minutes after gentle mixing. The tubes were heat-shocked for 5 minutes at 37° C. The transformed cells were transferred to glass tubes containing 20 μ l of 100 mM IPTG, 50 μ l of 2% X-Gal in dimethylformamide and 200 μ l of freshly grown DH5 α F' cells. To each tube, 3 ml of molten H top agar was added. The mixture was vortexed briefly and poured quickly onto a pre-warmed H agar plate. Once the top agar had set, plates were inverted and incubated at 37° C overnight.

2.15.5 Preparation of Single-stranded Template:

An overnight culture of DH5 α F', grown in LB, was diluted 1:100 into 100 ml of LB. The culture was grown at 37° C with vigorous shaking for 1 hour.

Aliquots of 1.5 ml of this culture were dispensed into sterile 20 ml disposable culture tubes. Using a toothpick, each tube was inoculated with a single colourless M13 plaque. One blue plaque was also inoculated as a control. Tubes were incubated for 6 hours at 37° C with aeration. Cultures were transferred to Eppendorf tubes. The cells were harvested by centrifugation in a microfuge for 5 minutes at room temperature. The supernatant was transferred to a fresh tube and re-centrifuged to ensure that all cells were removed.

To determine if the colourless plaques represented recombinants, a plaque assay was performed. Samples containing 20 μ l of supernatant, 1 μ l of 10% SDS and 4 μ l loading dye were electrophoresed on a 0.8% agarose gel run in 1x TBE buffer. The supernatant resulting from the inoculation of a blue plaque was used as a negative control, with increased electrophoretic mobility. Recombinants were then further processed.

To 1.2 ml of supernatant (the remaining supernatant was stored at 4° C) 250 μ l of 20% PEG-NACL (2.5 M NaCl with 20% w/v PEG 6000) was added. The tubes were mixed thoroughly by inversion and incubated at room temperature for 30 minutes. Precipitate was collected by centrifugation in a microfuge for 10 minutes. The supernatant was discarded and the tubes were spun for an additional 5 minutes. All traces of supernatant were removed and the viral pellet was resuspended in 100 μ l of TE buffer. The single stranded DNA was purified by phenol extraction and ethanol precipitation. The single stranded DNA pellet was resuspended in 20 μ l of TE buffer and stored at 4° C until further use.

2.15.6 Complementary Strand Test:

In an Eppendorf tube, 10 μ l of supernatant from a recombinant was combined with that of another recombinant in the presence of 1.5 μ l of 5M NaCl and 5 μ l of buffer C (60% formamide, 27% SDS and 0.1% bromophenol blue). As

negative controls, the supernatant from one of the recombinants was substituted with water. Tubes were incubated at 65° C for 45 minutes and cooled slowly to room temperature for 30 minutes. Tubes were centrifuged briefly in a microfuge and samples were electrophoresed on a 0.8% agarose gel run in 1x TBE buffer. Samples containing complementary strands were observed to have reduced electrophoretic mobility.

2.15.7 Sequencing Reactions:

Annealing, labeling, and termination reactions were carried out according to the protocol for the Sequenase Version 2.0 kit as described by the manufacturer (United States Biochemical Corporation, Cleveland, Ohio). Briefly, in an Eppendorf tube, purified single stranded DNA was combined with 0.5 pmoles of the desired primer (Table 2.3) in the presence of 5x sequenase reaction buffer to give a final volume of 10 μ l. The mixture was incubated at 65° C for 2 minutes and cooled slowly to 30° C. The labeling reaction was prepared by combining the annealed template-primer with 0.1M DDT, dGTP labelling mix, [^{35} S]ATP and sequenase. The mixture was centrifuged briefly in a microfuge and left at room temperature for 5 minutes. When the labelling reaction was complete, aliquots of 3.5 μ l were distributed to the rim of each of 4 tubes containing a respective ddNTP termination mix. Tubes were recentrifuged briefly and incubated at 37° C for 5 minutes. The termination reactions were stopped by the addition of 4 μ l of stop solution. Samples were stored at -20° C until required.

2.15.8 Preparation of a 6% Polyacrylamide gel for Sequencing:

The gel was prepared by dissolving 63 g of ultra pure urea in 53 ml of ddH₂O. To aid dissolution, the mixture was heated in a microwave oven for 1 minute. Once the solution had cooled, 0.234 g of bisacrylamide and 8.76 g of

Table 2.3 : List of oligonucleotide primers used.

Primer	DNA Sequence	Source/Reference
P ₁	5'-CAATGAGTTATATTAGCTCT	Kozlowski et al., 1987
P ₂	5'-TTGCTTACTGAAGGCTGTG	
P ₃	5'-AATTGAGCAAGACTTTGCAT	
P ₄	5'-AATTCGCCCATCTTCATGAC	"
P ₅	5'-AGGTATAATTAAAGTTATGG	"
P _A	5'-CGCGTTCTAGGGGTCGACCGAGCC	This study
P _B	5'-TCTATGGGATCCACCCACCTTAC	
P _C	5'-TGGTAGTGTAGAGAGTAA	
P _D	5'-GTTCTGCGGTAAGCTTTTACCCGA	"
P _E	5'-TATACCTTTGTGTATTTGTGGAT	"
P _F	5'-ATCAGCACACATGAGCCCTG	"
Universal	5'-GTAAACGACGCGCCAGT	USB Corporation (Cleveland, Ohio)

acrylamide was added and the mixture was stirred continuously. The solution was filtered through Whatman #1 filter paper into a graduate cylinder containing 30 ml of 5x TBE buffer. De-ionized distilled water (ddH₂O) was added to give a final volume of 142.5 ml. After cleaning and assembling the glass plates, the polyacrylamide gel was prepared for pouring by adding 60 μ l of TEMED and 7.5 ml of freshly prepared 0.75% ammonium persulfate to give a final volume of 150 ml. The solution was quickly mixed together and poured between the plates. A spacer was held within 1 cm on the gel by clamps and the gel was left to polymerize overnight.

2.15.9 Electrophoresis:

The gel was pre-electrophoresed at 700-900 volts (40 mA) for 30-45 minutes. Samples were prepared for loading by heating for 3 minutes at 95° C. Once 2 μ l of each sample was loaded, 1000-1300 volts (45 mA) was applied and maintained throughout the run.

2.15.10 Autoradiography:

Following electrophoresis, the plates were removed from the apparatus and left to cool for about 5 minutes. The top plate was removed and the gel, adhered to the bottom plate, was fixed for 20 minutes in 10% acetic acid with 10% methanol. The gel was transferred to Whatman 3MM paper and dried under vacuum at 80° C for 1 hour. The gel was autoradiographed overnight at room temperature using Kodak X-OMAT AR diagnostic film. The x-ray film was developed using an AEP Imaging 14XL x-ray film processor.

2.16 Other Procedures

2.16.1 Large Scale Isolation of Plasmid DNA:

An overnight culture, grown in LB containing the appropriate antibiotic depending on the plasmid used, was diluted 1:100 into 250 ml of LB and grown to stationary phase with vigorous shaking. The cells were harvested by centrifugation for 10 minutes at 6000 rpm. The supernatant was discarded and the cells were resuspended in 15 ml of cold TS buffer (50 mM Tris-Cl pH 8.3 with 25% w/v sucrose) and 5 ml of 0.25M EDTA pH 8.0. To the cell suspension, 2.5 ml of a 10 mg/ml lysozyme solution, freshly prepared using 0.25M EDTA pH 8.0 was added. The resuspended cells were mixed gently and incubated on ice for 10 minutes. The cells were lysed following the addition of 4 ml of Triton-X-lytic solution (50 mM Tris, 60 mM EDTA pH 8.0, and 2% v/v Triton-X-100) and incubation at 65° C for 5 minutes. The lysate was centrifuged for 60 minutes at 15000 rpm. DNA was precipitated from the supernatant using 1/3 volume of 40% PEG-NaCl (2M NaCl with 40% w/v PEG 6000/8000) at 4° C overnight.

The precipitate was collected by centrifugation at 6000 rpm for 10 minutes. The supernatant was discarded and the tubes were drained for 5 minutes. To the precipitate, 3 ml of TE buffer, 4.0 g of CsCl and 0.8 ml of 10 mg/ml ethidium bromide was added followed by a 15 minute incubation on ice. Using CsCl saturated TE buffer or TE buffer, the refractive index was adjusted to $1.392 \pm .002$. Beckman quickseal ultracentrifuge tubes were filled with solution, layered with mineral oil and sealed. The samples were centrifuged using a Beckman VTi80 rotor at 60000 rpm for 16 hours at 18° C.

Using a long-wave ultraviolet lamp, the DNA bands were visualized. The lower band, corresponding to covalently closed circular plasmid DNA was retrieved by side puncture of the tube with a 21 gauge needle, and transferred to a disposable culture tube. Ethidium bromide was removed from the plasmid

fraction by extracting thrice with 2 volumes of CsCl-saturated isopropanol. The DNA was dialyzed overnight against 1 litre of sterile TE buffer at 4° C. Following dialysis, plasmid DNA was isolated by ethanol precipitation using siliconized cover tubes. The precipitate was pelleted by centrifugation at 7000 rpm for 60 minutes. The supernatant was discarded and the precipitate was air dried. The plasmid DNA precipitate was redissolved in 50 μ l of TE buffer and stored in an Eppendorf tube at 4° C.

2.16.2 Rapid Extraction of Plasmid DNA by Boiling:

To an Eppendorf tube, 1.5 ml of an overnight culture, grown in LB with the appropriate antibiotic, was transferred. Cells were collected by centrifugation for 1 minute. The cell pellet was resuspended in 0.4 ml STET solution (10 mM Tris-Cl pH 8.0, 50 mM EDTA pH 8.0, with 5% v/v Triton-X-100 and 8% w/v sucrose) and 20 μ l of a 10 mg/ml freshly prepared solution of lysozyme. The tubes were placed on ice for 5 minutes. Lysis was accomplished by transferring the tubes to a boiling water bath for 45 seconds. The lysate was collected by centrifugation in a microfuge for 5 minutes. The gelatinous cellular debris was removed using a toothpick. Plasmid DNA was recovered from the supernatant by ethanol precipitation. The precipitate was resuspended in 20 μ l of TE buffer and 1 μ l of 10 mg/ml Rnase solution. The DNA suspension was incubated at 37° C for 10 minutes before storage at 4° C.

2.16.3 Transformation:

An overnight culture, grown in LB, was diluted 1:50 into 100 ml of LB. The culture was grown at 37° C with shaking to early log phase. The culture was then placed on ice for 20 minutes. The cells were harvested by centrifugation at 6000 rpm for 10 minutes at 4° C using 2 SS34 tubes. The cell pellet was pooled and

resuspended using 15 ml of cold 0.1M MgCl_2 . The cells were collected by centrifugation as before. The pellet was resuspended in 2 ml of cold 0.1M CaCl_2 and incubated on ice for a minimum of 1.5 hours. In Eppendorf tubes, 200 μl of competent cells were added to each DNA sample and kept on ice for 30 minutes after gentle mixing. The cells were heat-shocked for 5 minutes at 37° C. To each tube, 800 μl of LB was added and the tubes were re-incubated at 37° C for 1 hour. On selective media, 0.1 ml, 0.3 ml and 0.6 ml of transformed cells were plated.

2.16.4 Restriction Digestion of Plasmid DNA:

Approximately, 1 μg of CsCl purified plasmid DNA or 5 to 10 μl of rapid extracted plasmid DNA was digested in a 20 μl volume using 5 units of restriction enzyme with TA buffer for 90 minutes. If the digest was to be electrophoresed, 1/5 volume of stop solution was added. In other cases, digested plasmid DNA was ethanol precipitated before further treatment.

2.16.5 Ligations:

Approximately 1 unit of T4 DNA ligase was added to a 10 μl reaction volume in the presence of 5x ligase buffer. Ligations were carried out at 14° C overnight. Half of the ligation mix was used per transformation.

2.16.6 Phenol Extractions:

Samples were diluted with TE buffer to a volume of 200 μl . An equal volume of phenol, saturated with TE buffer, was added and samples were vortexed to form an emulsion. The phases were separated by centrifugation for 3 minutes in a microfuge. The upper aqueous layer was transferred to a fresh Eppendorf tube. A back extraction was performed by adding an equal volume of TE buffer to the organic phenol layer, vortexing the mixture and centrifuging as before. The

resulting aqueous layer was also transferred to the Eppendorf tube. An equal volume of chloroform: isoamyl alcohol (24:1) was added to the combined aqueous layers. The mixture was vortexed briefly and centrifuged once more for 3 minutes. The upper aqueous phase was removed, carefully avoiding material at the interface, and transferred to a fresh Eppendorf tube for ethanol precipitation.

2.16.7 Ethanol Precipitation:

To the supernatant, 1/5 volume of 10M ammonium acetate was added and mixed thoroughly by tapping, followed by the addition of 2.5 volumes of cold absolute ethanol. The solution was mixed by inversion and incubated on ice for 15 to 60 minutes. The precipitate was collected by centrifugation in a microfuge for 10 minutes. The supernatant was removed and the pellet was washed with 1 ml of absolute ethanol. Centrifugation was repeated as before for 3 minutes and the pellet was air dried prior to resuspension in the appropriate volume of TE buffer.

2.16.8 Agarose Gel Electrophoresis:

Agarose gels were prepared by dissolving 0.7, 0.8 or 1.0% (w/v) agarose in 1x TBE buffer or 1x TAE buffer. The agarose was dissolved with occasional swirling, in the microwave oven. Before pouring, the solution was cooled briefly in a 45° C water bath. After pouring, the appropriate comb was placed in the gel and was left to solidify for at least 15-20 minutes. Samples were electrophoresed in the appropriate buffer (1x TBE or 1x TAE) at 100 volts until the bromophenol blue dye front reached the bottom of the gel. After a 10 minute staining period in a 1 μ g/ml solution of ethidium bromide and destaining in water for 20 minutes, the gel was illuminated under UV light and photographed using Polaroid type 57 film.

3.0 RESULTS

3.1 Isolation of Copy Mutants of pCU32:

Plasmid pCU32 is a 6 kb miniplasmid derivative of pCU1 containing the 2 kb replicon region and the gene that confers resistance to the antibiotic, ampicillin (Ap^R) (Figure 1.0). Copy mutants of pCU32 were isolated in the presence of elevated levels of ampicillin as it has been shown that resistance to increasing concentrations of some antibiotics, such as ampicillin, is proportional to the number of copies of the resistance gene present in the bacterial cell (Uhlen and Nordstrom, 1977). In some cases, medium containing elevated levels of ampicillin was supplemented with clavulanic acid, a potent β -lactamase inhibitor that reduces the MIC of ampicillin by about 50-fold (Barth, 1987). As a result, two classes of spontaneous copy mutants were obtained, depending on the presence or absence of clavulanic acid, at a frequency of 10^{-6} to 10^{-7} per colony-forming unit, respectively. Two of these mutants were chosen from each class for further study.

Class I copy mutants, pCU32 *cop3* and *cop75*, independently isolated on LA plates containing elevated levels of ampicillin only expressed an approximate 2-fold and 1.5-fold increase in the MIC of ampicillin necessary to prevent cell growth, respectively (Figure 3.0). Class II copy mutants, pCU32*copI* and *copL*, isolated on LA plates containing elevated levels of ampicillin and clavulanic acid, expressed a 5-fold and 4.5-fold increase in the MIC of ampicillin respectively (Figure 3.0). The possibility of mutation to the antibiotic resistance gene promoter was eliminated by the observed change in the amount of plasmid DNA of the mutants following agarose gel electrophoresis (Figure 3.1). Copy number change, resulting from plasmid DNA alteration and not from host chromosomal mutations, was verified by the maintenance of increased resistance to ampicillin by *E. coli* HB101 cells re-transformed with copy mutant plasmid DNA.

Figure 3.0 : Ampicillin resistance of *E.coli* HB101 cells carrying (●) pCU32,, (▲) pCU32*cop*3, (△) pCU32*cop*75, (■) pCU32*cop*I and (◻) pCU32*cop*L.

Figure 3.1 : Agarose gel electrophoresis of pCU32 and its copy mutants. The DNA was prepared by the modified Eckhardt procedure as described in Materials and Methods. The whole cell lysates were electrophoresed on a 1.0% agarose-TBE gel. The gel was stained with EtBr, photographed and the negative was scanned using a densitometer to determine copy number. The arrow marked as Chr refers to chromosomal DNA, the other arrow indicates plasmid DNA. Lane 1, pCU32/HB101; Lane 2, pCU32*cop*75/HB101; Lane 3, pCU32*cop*3/HB101; Lane 4, pCU32*cop*I/HB101; Lane 5, pCU32*cop*L/HB101; Lane 6, empty; Lane 7, HB101.

3.2 Determination of Copy Number:

Plasmid copy number was determined using two methods as described in Materials and Methods, Section 2.10. When possible, plasmids of known copy number were used as standards for each determination to promote confidence in the values obtained. Plasmid pCU32, the wild-type plasmid used for isolating copy mutants was found to have a copy number of about 37, 2-fold greater than its parental plasmid, pCU1, from which it was derived (Table 3.0). Class I copy mutants, pCU32*cop3* and *cop75*, were found to have a 2-3 fold increase in copy number as compared to pCU32 (Table 3.0). Class II copy mutants, pCU32*copI* and *copL*, were found to express copy numbers 5-fold greater than pCU32 (Table 3.0).

3.3 Isolation of the Copy Mutant Replicon:

To determine if the copy mutation(s) were present in the 2 kb replicon region of the pCU32-derived copy mutant plasmids, the respective 2 kb regions of the mutant plasmids were isolated and linked to the kanamycin resistance gene from the vector pLG338 by shot-gun cloning (Figure 3.2). Constructed plasmids harboring the Class I copy mutant replicons were designated as pMN*cop3* and pMN*cop75* corresponding to pCU32*cop3* and pCU32*cop75* respectively. Recombinants harboring the class II copy mutant replicons were denoted as pMN*copI* and pMN*copL* corresponding to pCU32*copI* and pCU32*copL* respectively. A mini-pCU32 derivative containing only the 2 kb wild-type replicon linked to the kanamycin resistance gene fragment of pLG338 was designated as pMN32. Compared to *E. coli* HB101 cells carrying pMN32, HB101 cells harboring plasmids carrying the copy mutant replicons displayed increased resistance to kanamycin (Table 3.1). Class I copy mutants, pMN*cop3* and *cop75*, expressed approximately a 3-fold and 2-fold increase in the MIC of kanamycin, respectively

Table 3.0: Comparison of copy numbers of PCU32 and its copy mutants.

Plasmid	Class ^a	Antibiotic Resistance ^b	Plasmid Size (Kb)	Plasmid Copies/Cell	
				Method 1 ^c	Method 2 ^d
PCU1	-	Ap, Sp, Sm	3.9	-	15.2±2.2
PCU32	-	Ap	6	41.2±5.2	34.3±4.0
PCU32GCB3	I	Ap	6	125.2±17.2	113.5±11.8
PCU32GCB75	I	Ap	6	87.1±10.2	90.4±7.6
PCU32GCB1	II	Ap	5.6	203.4±35.1	186.1±19.8
PCU32GCB1	II	Ap	5.6	195.1±24.4	160.3±14.7
PMN32 ^h	-	Km	3.7	-	44.4±5.6
PMNGCB31	I	Km	3.7	-	105.1±10.9
PMNGCB751	I	Km	3.7	-	94.2±6.8
PMNGCB11	II	Km	3.3	-	174.8±15.1
PMNGCB11	II	Km	3.3	-	149.4±13.9
PMNGCB11	II	Km	4.3	-	52.9±4.9
PBR322	-	Ap, Tc	56.4	-	6.2±1.6
RK2	-	Ap, Km, Tc	38	-	15.5±3.2
R6K	-	Ap, Sm	38	-	15.5±3.2

The values reported are mean values of at least 3 independent determinations carried out in duplicate with their standard deviations.

^aIndicates isolation of copy mutant in the absence (I) or presence of clavulanic acid (II).

^bIndicates resistance to ampicillin (Ap), spectinomycin (Sp), streptomycin (Sm), kanamycin (Km) and tetracycline (Tc).

^cCopy number determined by gel electrophoresis of whole cell lysates as described in Materials and Methods.

^dCopy number determined using ³H-thymidine incorporation as described in Materials and Methods.

^e,1,1,9These plasmids have reported copy numbers of 35-50 copies/cell, 4.7 copies/cell and 15-20 copies/cell respectively.

^h1Derivatives harboring wild-type or copy mutant replicons respectively (see Figure 3.2).

Figure 3.2 : Construction of plasmids carrying wild-type (pMN32) or copy mutant replicons (pMN*cop*3, pMN*cop*75, pMN*cop*I and pMN*cop*L) as described in Materials and Methods. The pCU32 or copy mutant replicon is indicated by a thick line. A hatched line indicates the replicon of pLG338. Antibiotic resistance genes, Ap^R, Tc^R and Km^R are indicated by thin lines representing ampicillin, tetracycline and kanamycin respectively; Pv, restriction site for PvuII.

Table 3.1 : Growth of *E. coli* HB101 cells harboring plasmids carrying wild-type and copy mutant replicons on LA media containing various concentrations of kanamycin.

Plasmid used	Concentration of Kanamycin ($\mu\text{g/ml}$)							
	0	500	1000	1250	1500	2000	4000	6000
PMN32	+	+	+	-	-	-	-	-
PMN _{cop3}	+	+	+	+	+	+	-	-
PMN _{cop75}	+	+	+	+	+	-	-	-
PMN _{copI}	+	+	+	+	+	+	+	+
PMN _{copL}	+	+	+	+	+	+	+	+

(Table 3.1). Class II copy mutants, pMN*copI* and *copL*, displayed at least a 6-fold increase in their level of resistance to kanamycin (Table 3.1). Using ^3H -thymidine incorporation, the copy number of these recombinants was found to be similar to pCU32-derived copy mutant plasmids confirming that copy number changes were a result of alterations to the respective replicons (Table 3.0).

3.4 Mapping the Copy Mutations

3.4.1 Restriction Digestion:

Restriction digestion of copy mutants was used to identify putative point mutants from those that had undergone massive rearrangements. The location and nature of these changes was precisely mapped by sequencing. The mobility of restriction fragments, generated by the restriction endonucleases PvuII and HaeII, of Class I copy mutants and wild-type plasmid pCU32 were found to be identical (Figure 3.3). Although five fragments were expected to be generated by PvuII digestion of pCU32 (Figure 3.2) only three PvuII fragments were resolved as the two smaller fragments had run off the gel under the conditions used here. HaeII digestion was expected to result in the generation of a number of small fragments, many of which also have run off the agarose gel (Figure 3.3). However, unlike PvuII, there are a number of HaeII sites within the 2 kb replicon, particularly within the Group I repeats. As the digestion patterns of the Class I mutants and wild-type were identical, elevated copy number of these mutants was attributed to point mutations or small deletions within the 2 kb replicon region. Conversely, the restriction pattern of the Class II copy mutants was observed to be identical to each other but dissimilar to pCU32 (Figure 3.3). As a result of PvuII digestion, only one 5.6 kb fragment was generated from pCU32*copI* and *copL* indicating the loss of some of the PvuII sites, a result attributed to sequence rearrangement within these plasmids. A similar reorganization of HaeII sites was detected

Figure 3.3 : Agarose gel electrophoresis of restriction fragments of pCU32 and its copy mutants. Plasmid DNA was prepared from cultures grown to a standard density using the rapid extraction procedure as described in Materials and Methods, linearized with PvuII and HaeII and electrophoresed on a 0.8% agarose-TBE gel. Lane 1, pCU32; Lane 2, pCU32/PvuII; Lane 3, pCU32/HaeII; Lane 4, pCU32*cop3*; Lane 5, pCU32*cop3*/PvuII; Lane 6, pCU32*cop3*/HaeII; Lane 7, pCU32*cop75*; Lane 8, pCU32*cop75*/PvuII; Lane 9, pCU32*cop75*/HaeII; Lane 10, pCU32*copI*; Lane 11, pCU32*copI*/PvuII; Lane 12, pCU32*copI*/HaeII; Lane 13, pCU32*copL*; Lane 14, pCU32*copL*/PvuII; Lane 15, pCU32*copL*/HaeII; Lane 16, 1 kb MW standard DNA.

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

8
5
4
3.0
2.0
1.6
1.0
.51, .52

suggesting that some sequence rearrangement and/or deletion had occurred, most likely within the Group I repeats.

3.4.2 Incompatibility:

Plasmid pCU32 expressed strong incompatibility with the mini-pCU32 derivative, pMN32 (Table 3.2). Similar incompatibility was expressed by the Class I copy mutants suggesting that these copy mutations are not associated with the incompatibility functions expressed by the replicon (Table 3.2). The Class II copy mutants were also found to express incompatibility, marginally reduced, towards pMN32 (Table 3.2). The resident plasmid, pMN32, was found to be stably maintained in the absence of any donor plasmid (Table 3.2).

3.4.3 Trans-Complementation:

The presence of pRI114, a derivative of the 2 kb replicon carrying the Group II direct repeats and ORF₄₆ (Figure 3.4) in *trans*, had no effect on the level of resistance to kanamycin expressed by the wild-type replicon (pMN32) or the copy mutants, pMN*cop*3, pMN*cop*I and pMN*cop*L (Table 3.3). Increased kanamycin resistance expressed by pMN*cop*75 was reduced slightly in the presence of pRI114 in *trans*. The level of kanamycin resistance expressed by wild-type and copy mutant replicons was not reduced in the presence of pRI123 (Figure 3.4), a cloned derivative harboring only 1 of the Group I direct repeats (Table 3.3). The presence of all the Group I repeats in *trans*, on pRI105 (Figure 3.4), did reduce the level of kanamycin resistance expressed by both classes of copy mutants whereby the most prominent reductions were expressed by the Class II copy mutants, pMN*cop*I and *cop*L (Table 3.3). A corresponding reduction in plasmid DNA yield and hence copy number of pMN*cop*I and *cop*L was observed (Figure 3.5). However, only the copy number of the Class I copy mutant, pMN*cop*3, was

Table 3.2 : Incompatibility of wild-type PCU32 and its copy mutants with PMN32.

Plasmid		Percent loss of	
Donor	Resident	Resident Plasmid ^a	Donor Plasmid ^a
PCU32	PMN32	100	0
PCU32 ~~cop~~ 3	"	100	0
PCU32 ~~cop~~ 75	"	100	0
PCU32 ~~cop~~ I	"	90	0
PCU32 ~~cop~~ L	"	94	0
-	"	0	-

The values reported are mean values of 2 independent determinations.

^aPercent loss of the resident or donor plasmid was determined by calculating the fraction (%) of colonies that failed to grow on media containing the resident or donor antibiotic marker drug.

Figure 3.4 : Summary of the physical and genetic map of pCU1 and its miniplasmid derivative shown with the structural organization of its 2 kb replicon (thick solid bar) as described in Figure 1.0. The altered replicons of isolated copy mutants (pCU32*cop3*, *cop75* and *copI*) are shown below the salient features of the replicon. Vertical arrows indicate the location of point mutations, a gapped line represents a deletion. pCU32*copL* is not shown as it was not sequenced. However, it is inferred to have a deletion located at a region similar to that shown for pCU32*copI*. Derivatives of pBR322 carrying different regions from the pCU1 replicon are also shown (pRI114, pRI123, pRI105 and pRI119). Abbreviations: Sp^r, Sm^r and Ap^r, regions determining resistance to spectinomycin, streptomycin and ampicillin, respectively; ORF, open reading frame; A, *Ava*I; B, *Bgl*II; Pv, *Pvu*II; and Sma I.

Table 3.3 : Effect of various deletion derivatives on the degree of kanamycin resistance of *E.coli* HB101 cells harboring plasmids containing the wild-type or copy mutant replicons.

Plasmid		pCU1 Replicon Coordinates	Degree of Kanamycin
Donor	Resident	of Donor ^a	Resistance (μ g/ml)
-	pMN32	-	1000-1250 ^b
pBR322	"	-	"
pRI105	"	1374-2053	"
pRI114	"	1-1016	"
pRI119	"	1017-1593	250-500
pRI123	"	1825-2053	1000-1250
-	pMNCop3	-	2000-4000 ^b
pBR322	"	-	2000-4000
pRI105	"	1374-2053	1250-1500
pRI114	"	1-1016	2000-4000
pRI119	"	1017-1593	500-750
pRI123	"	1825-2053	2000-4000
-	pMNCop75	-	1500-2000 ^b
pBR322	"	-	1500-2000
pRI105	"	1374-2053	1250-1500
pRI114	"	1-1016	1250-1500
pRI119	"	1017-1593	250-500
pRI123	"	1825-2053	1500-2000
-	pMNCopI	-	>6000 ^b
pBR322	"	-	>6000
pRI105	"	1374-2053	1000-1250
pRI114	"	1-1016	>6000
pRI119	"	1017-1593	500-750
pRI123	"	1825-2053	>6000
-	pMNCopL	-	>6000 ^b
pBR322	"	-	>6000
pRI105	"	1374-2053	1000-1250
pRI114	"	1-1016	>6000
pRI119	"	1017-1593	500-750
pRI123	"	1825-2053	>6000

The values reported are a result of 3 independent determinations by replicating 50 transformants onto LA plates selecting for donor plasmid and increasing resistance to kanamycin, conferred by the resident plasmid.

^aSee Figure 2.0.

^bSee Table 3.1.

Figure 3.5 : Effect of various deletion derivatives on the copy number of mini-pCU32 and its copy mutants. Co-resident plasmid DNA was prepared from cultures grown to a standard density using the rapid extraction procedure as described in Materials and Methods and electrophoresed on a 0.7% agarose-TBE gel. Lane 1, pMN32; Lane 2, pMN*cop*75; Lane 3, pMN*cop*3; Lane 4, pMN*cop*I; Lane 5, pMN*cop*L; Lane 6, pRI105; Lane 7, pRI105/pMN32; Lane 8, pRI105/pMN*cop*75; Lane 9, pRI105/pMN*cop*3; Lane 10, pRI105/pMN*cop*I; Lane 11, pRI105/pMN*cop*L; Lane 12, pRI119; Lane 13, pRI119/pMN32; Lane 14, pRI119/pMN*cop*75; Lane 15, pRI119/pMN*cop*3; Lane 16, pRI119/pMN*cop*I; Lane 17, pRI119/pMN*cop*L; Lane 18, 1 kb MW standard DNA.

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

6.1
5.1
4.1
3.1
2.0

observably affected (Figure 3.5). A dramatic reduction in kanamycin resistance was observed for Class I and Class II copy mutants when ORF₆₈ and the first 6 Group I direct repeats were provided in *trans* by pRI119 (Figure 3.4). A similar reduction in resistance to kanamycin, expressed by the wild-type replicon pMN32, was also observed when pRI119 was provided in *trans*, suggesting the presence of a strong trans-acting inhibitor. A corresponding reduction in plasmid copy number of this mini-pCU32 derivative and Class I and II copy mutants was detected using agarose gel electrophoresis (Figure 3.5). The presence of pBR322, the vector used to construct the deletion derivatives, had no effect on the expression of kanamycin resistance.

3.4.4 Nucleotide Sequencing of Class I and II Copy Mutants:

3.4.4.1 Class I Mutants:

DNA sequencing of the 2 kb replicon of pCU32*cop3* revealed a consecutive 3 base substitution from CGA to GAC starting at position 1039 within ORF₂₃₉ (Figure 3.6a). This results in a single amino acid change from aspartic acid (GAT) to threonine (ACT). Although the preceding codon is altered from CGC to CGG, the amino acid, aspartic acid, remains unchanged. Sequencing of the pCU32*cop75* replicon disclosed a number of mutations within the first 300 bp (Figure 3.7). As well, a single base substitution from T to G was detected at position 1148 within ORF₂₃₉ and ORF₆₈ (Figure 3.6b). This results in a single amino acid alteration from phenylalanine (TTC) to valine (GTC).

3.4.4.2 Class II Mutants:

DNA sequencing of the pCU32*copI* replicon disclosed a massive deletion of 372 bp within the Group I repeats so that only 3 repeats remain (Figure 3.8). Unique nucleotides within the first 2 repeats and the last repeat were mapped to

Figure 3.6 : Nucleotide sequences containing point mutations of (A) pCU32*cop3* and (B) pCU32*cop75*. Asterisks indicate site of mutation(s).

A

B

Figure 3.7 : DNA sequence analysis of pCU32*cop75* containing multiple mutations between nucleotides (A) 154-185 and (B) 275-285. Asterisks mark altered nucleotides.

Figure 3.8 : Nucleotide sequence containing a deletion of the Group I direct DNA repeats of pCU32*copI*. Asterisks indicate unique nucleotides used for identification of the remaining repeats.

pCU32*copI*. No other mutation within this copy mutant was found. The copy mutant, pCU32*copL*, was not sequenced. However, a similar sequence alteration was expected as, for the enzymes used, the restriction pattern of its DNA was similar to pCU32*copI* (Figure 3.3). The alterations in the DNA sequence of the copy mutant replicons revealed by nucleotide sequencing, are summarized in Table 3.4 and Figure 3.4.

3.4.5 PolA Dependence:

Both classes of copy mutants and the wild-type plasmid, pCU32, were capable of conferring antibiotic resistance on their *polA*⁻ hosts indicating that they replicate by a PolA independent mode of replication (Table 3.5). The PolA dependent plasmid, pBR322, and the PolA independent plasmid, pLG338, behaved as expected in the *polA*⁻ host, SR1672 (Table 3.5). SR1758, an isogenic *polA*⁺ strain, was able to grow on selective media when transformed with any of the test plasmids (Table 3.5).

Table 3.4 : Summary of sequence alteration to the 2 kb copy mutant replicons.

Copy Mutant	Position ^a	Wild-type	Mutation	Amino Acid Substitution ^b
<u>cop3</u>	1039-1041	CGA	GAC	Asp → Thr
<u>cop75</u>	1148	T	G	Phe → Val
	158	C	G	
	159	G	C	
	165-170	GGCGTTG	GGGCGTGGC	
	180-185	CCGAAG	CCGAA GC	
	276-279	AGTG	AGCGGCGCTG	
<u>cop1</u>	1367-1862	13 Group I Repeats	3 Group I Repeats	

^aSee Figure 1.0

^bIndicates an amino acid substitution of aspartic acid (Asp) to threonine (Thr) and phenylalanine (Phe) to valine (Val).
 Underlined bases or gapped spaces represent altered, added or deleted nucleotides.

Table 3.5 : Transformation of a polA⁻ mutant strain and its polA⁺ isogenic strain with PCU32 and its copy mutants.

Plasmid	SR1672 (polA Null Mutant)	SR1758 (Isogenic polA ⁺ strain)
PCU32	+	+
PCU32COP3	+	+
PCU32COP75	+	+
PCU32COP1	+	+
PCU32COPL	+	+
PBR322	-	+
PLG338	+	+

^A + symbol indicates growth of approximately 150-200 transformants using 0.5 µg of plasmid DNA.

4.0 DISCUSSION:

In this study, a number of spontaneously acquired mutations which increase copy number of pCU32, a deletion derivative of pCU1, have been identified: deletions to the Group I direct DNA repeats, base substitutions in ORF₂₃₉ and ORF₆₈ and multiple mutations between nucleotides 1-300. By relating sequence alterations to functional changes, it has become possible to assess the significance of the altered elements in copy number control and by doing so, propose a mechanism(s) of regulation of replication.

4.1 Group I Direct DNA Repeats:

The role of direct repeats as titrators of the replication initiator, or Rep protein, has been well documented in the literature. From the results presented here, the Group I direct repeats appear to play a similar role in regulating the rate of replication of pCU32. DNA sequencing revealed a deletion of 10 direct repeats in the class II copy mutant, pCU32*copI*, that may have occurred via a recombinational event between the third and thirteenth repeat (Figure 3.8). The loss of the majority of the Group I repeats from *copI* resulted in a 5-fold increase in copy number (Table 3.0). Expectedly, the loss of the direct repeats also resulted in reduced expression of incompatibility due to the inability of the co-resident plasmid, pCU32*copI*, to compete for elements of plasmid replication like Rep, a protein necessary for replication (Table 3.2). These observations and interpretations are consistent with the observed properties of pCU751, a deletion derivative of the 2 kb region lacking the Group I direct repeats (Krishnan, 1989). Elevated copy number, could be suppressed by providing the repeats in *trans* indicating that these repeats exhibit a negative influence on copy number by effectively inhibiting replication perhaps by titration of the replication initiator (Table 3.3). A similar effect was also shown for plasmid P1 whereby copy mutants,

resulting from a loss of direct repeats, appeared sensitive to the repeats when provided in *trans* (Pal et al., 1986). The other Class II copy mutant, pCU32*copL*, is also likely to carry a deletion within the Group I direct repeats as the *Hae*II restriction digests of both Class II copy mutants, an endonuclease that has a number of sites within the Group I repeats, are identical (Figure 3.3). As well, *copL* has a high copy number and has lost some incompatibility function (Tables 3.0 and 3.2). However, the number of repeats present in *copL* may be different from those present in *copI* which would give evidence to the gene dosage effect of the repeats in controlling copy number.

Wild-type copy number was also reduced when the first 6 Group I direct repeats were provided in *trans* by pRI119 suggesting that there may be some specificity among the Group I direct repeats (Figure 3.5). Specificity among direct DNA repeats had also been proposed for plasmid P1 where the Rep protein expresses the highest affinity for the repeats in the *rep* gene operator, and those involved in copy number control and the lowest affinity for the repeats in the origin (Chattoraj et al., 1985). However, plasmid P1 Rep has not been found to have any particular affinity for individual repeat sequences (Abeles et al., 1989). As no copy number change was experienced by pMN32 in the presence of pRI105, a deletion derivative harboring all of the Group I direct repeats, some other element present on pRI119, such as ORF₆₈, is proposed to be responsible for this observation and is discussed in more detail in Section 4.3 (Figure 3.4). Nevertheless, to determine if some of the Group I direct repeats have a greater inhibitory ability than others by expressing a higher affinity for the replication initiator, or Rep protein, isolation of this protein and footprinting experiments with it would be required. Such studies would also confirm the role of the repeats in limiting the replication initiator.

4.2 ORF₂₃₉ (*rep* gene):

In this study, two kinds of mutations thought to affect copy number control of pCU32 were identified in the region of the *rep* gene, ORF₂₃₉, by DNA sequencing of the class I copy mutants. They resulted in an alteration by substitution of a single amino acid: aspartic acid to threonine at position 149 for pCU32*cop3* and, phenylalanine to valine at position 185 for *cop75* (Table 3.4). However, it should be noted that this mutation, in *cop75*, occurs in a region where ORF₂₃₉ and ORF₆₈ overlap (Figure 3.4). Although there may be some structural changes as a result of the amino acid substitutions, neither mutation inactivated the Rep protein as both copy mutants maintained a *polA* independent mode of replication (Table 3.5). The location of the mutations however, appears to be significant as both copy mutants expressed a 2 to 3-fold increase in copy number as compared to the wild-type, plasmid pCU32 (Table 3.0). Trans-complementation, using pRI105, appeared to have no effect on *cop75* (Figure 3.5). This may indicate that the *cop75* Rep protein remained unaffected by the amino acid substitution as a result of no change to the charge of the substituted amino acid side group. On the other hand, the *cop75* mutation may, in fact, affect ORF₆₈ only and so, is discussed in more detail in section 4.3. Increased copy number expressed by *cop3* could be suppressed slightly by providing the Group I direct repeats in *trans* using pRI105 (Figure 3.5). This finding suggests that the titration of the mutated *cop3* Rep protein by its own Group I direct repeats is inefficient.

This loss of copy number control, in pCU32*cop3*, could be attributed to the lowered binding affinity of the mutated Rep protein to the Group I direct repeats. As a result, the Group I direct repeats would be ineffective in limiting the amount of Rep protein from the system so that more initiator would be available for origin activation. The effect of providing more direct repeats in *trans* would likely compensate for this inefficient interaction and so copy number would be

observably reduced as the mutated Rep protein is titrated. A similar explanation has been suggested for the mutated Rep protein of the plasmid Rts1 (Terawaki and Itoh, 1985). Footprinting studies using the wild-type Rep protein and the Rep protein isolated from pCU32*cop3* would determine if the binding efficiency of the mutated initiator has indeed altered. However, it should be noted that the incompatibility expressed by pCU32*cop3* and *cop75* is similar to that expressed by the wild-type replicon indicating that co-resident plasmids are equally capable of competing for replication elements, like Rep (Table 3.2). This suggests that the loss of copy number control may not be a result of reduced binding affinity.

By contrast, the loss of copy number control in pCU32*cop3* could also result from the abolishment of repression of the *rep* gene. This implicates the Rep protein in having dual roles in the replication of pCU32: (i) as an initiator of replication and, (ii) as a negative regulator of its own expression. Thus, as a result of the *cop3* mutation, the mutated Rep protein would no longer be able to repress its own synthesis efficiently and so more protein would become available to activate the origin. As a consequence, the Group I direct repeats would be insufficient to limit the excess mutated Rep protein, an effect that could be reversed by providing more direct repeats in *trans*. Analysis of copy mutants resulting from base substitutions within the *rep* gene of plasmids F, P1 and R6K has shown that these respective replication proteins have a similar role (Bex *et al.*, 1986; Kelley and Bastia, 1985; Chatteraj *et al.*, 1985a). The location of the *cop3* mutation may be significant in deducing the regions of the Rep protein involved in autoregulation function. However, these results provide only some initial evidence of the Rep protein, encoded by ORF239, playing a direct role in copy number control itself. Footprinting experiments would be required to confirm binding of this initiator to its promoter.

4.3 ORF₆₈:

Another mechanism of control, initially displayed by trans-complementation analysis may be exhibited by the product of ORF₆₈. In these experiments, wild-type copy numbers were observably reduced in the presence of pRI119, a deletion derivative harboring 6 Group I direct repeats and ORF₆₈ (Figure 3.5). As no effect on wild-type copy number was observed when all the repeats (but not ORF₆₈) were provided in *trans*, these findings suggest that ORF₆₈ may encode a small trans-acting inhibitor. A single base substitution at position 1148 in pCU32*cop*75, a mutant expressing a 2-fold increase in copy number, was found in a region where ORF₂₃₉ and ORF₆₈ overlap. It is tempting to speculate that as a result of the mutation, the ability of the product of ORF₆₈ to inhibit initiation of replication is reduced. Trans-complementation using pRI119, did reduce the copy number of pCU32*cop*75 (Figure 3.5). However, as stated earlier, the effect of this derivative on wild-type copy number is also drastic, making it difficult to discern if complementation or trans-acting inhibition is occurring. A single base substitution in the gene of *copB* was also reported recently for the Inc FII group plasmid, R1 (Banerjee *et al.*, 1990). As a result, the small protein, CopB, was unable to efficiently repress transcription by binding to the promoter of the replication gene.

ORF₆₈ may also encode a small protein with similar copy number control activity as CopB. It should be noted that the single base substitution in ORF₆₈ occurs in the third position of the codon and so does not result in a subsequent change in the amino acid. However, it is possible that the inhibitor, encoded by ORF₆₈, is a RNA transcript that regulates copy number by interacting with the complementary Rep mRNA, transcribed in the opposite direction from ORF₂₃₉, thereby preventing complete translation of the replication initiator. A similar mechanism for inhibition of replication, by preventing the synthesis of the Rep

protein, has been reported for plasmids R1 and pT181 whereby countertranscript interaction prevents translation by destabilizing the *rep* message or by sequestering the ribosomal binding site, respectively (Wagner *et al.*, 1988; Carleton *et al.*, 1984). Loss of copy control by pCU32*cop*75 may be indicative of the importance of the base substitution for altered interaction between countertranscripts. A role of ORF₆₈ in plasmid replication has also been proposed by Krishnan (1989). To pursue its role as a copy number control element further, the inhibitor encoded by ORF₆₈ would have to be isolated.

4.4 Nucleotides 1-300:

It should be noted that multiple mutations were also found in the first 300 bp of the 2 kb replicon in pCU32*cop*75. The only structure that may be directly affected by this sequence alteration is a *dnaA* box at position 298. *dna* boxes are sites where the DnaA protein is thought to bind and induce initiation of replication in *E. coli* (Thomas, 1988). However, providing the *dnaA* box is functional and so is affected by sequence alteration, copy number would be expected to decrease, not increase as it has. It is possible however, that the multiple mutations have somehow destabilized immediate upstream structures such as the Group II repeats or ORF₄₆, a gene that has been shown to express a small trans-acting polypeptide (Krishnan, 1989). The downstream mutations appear to be somewhat complementable in *trans* by pRI114, a deletion derivative harboring the Group II repeats and ORF₄₆ (Figure 3.4; Table 3.3). As there is no effect by pRI114 on any of the other copy mutants, this result suggests that the effect of these mutations can be suppressed in *trans*. However, before the effect of these downstream mutations on copy number can be determined, the replicon of pCU32*cop*75 has to be recloned so that both mutations, the downstream multiple mutations and the base substitution in ORF₆₈, can be studied separately.

4.5 Other Copy Number Control Elements:

Broad-host-range, multicopy plasmids generally utilize a complex mechanism for controlling copy number. Often there are other factors determined by plasmid regions outside the basic replicon, like *korA*, *incC* and *korB* in RK2, that indirectly affect copy number. Such a possibility may also exist for pCU1 and so may be responsible for the difference in copy number between pCU1 and its miniplasmid derivative, pCU32.

4.6 Inverted Repeat Sequences:

Attention should also be drawn to the existence of a 19 bp sequence overlapping a Group II repeat member, which is present in inverted orientation straddling each Group I repeat member (Figure 1.0). As a result, potential for intrastrand hairpin loop formation exists that may influence regulation of ORF46 and ORF239 expression (Krishnan, 1989). However, evidence has yet to be obtained to ascribe a role for them in replication control.

4.6 Conclusion:

The procedure of selecting for increased ampicillin resistance with and without clavulanic acid has proved to be a useful way of obtaining copy number mutants of pCU32. From the analysis of the two classes of copy mutants obtained, it is tempting to speculate that the presence of clavulanic acid favors deletions and selection for small increases in ampicillin resistance, in the absence of clavulanic acid, favors point mutations. However, more copy mutants would have to be isolated using these two selective methods and analyzed before any firm conclusions can be made.

Comparing all the copy mutants isolated, the loss of the majority of the Group I direct DNA repeats appears to have had the most significant effect on plasmid copy number. However, the rate of replication and so, copy number, remains controlled albeit at a higher steady state. Likewise, adding more repeats could not reduce the protein concentration to a point at which no replication could occur. This suggests that more than one factor is involved in copy number control.

Based on these findings, the following mechanisms for regulating the rate of replication are proposed. The Group I direct DNA repeats control plasmid copy number by titrating the replication initiator, Rep, and so limiting its availability for activating the origin. There is some evidence to suggest that there may be a relationship of Rep affinity for the direct repeats which, when abolished, diminishes the negative effect of the direct repeats so that more protein is available for replication. However, Rep may be directly involved in the negative control of pCU32 copy number by autoregulation in addition to its absolute requirement for initiation of replication. Similarly, a small trans-acting inhibitor encoded by ORF₆₈, is suspected of inhibiting initiation by preventing translation of the Rep mRNA transcript. It should be noted however, that although the majority of mechanisms proposed here are based on the analysis of results presented in this study, they do provide a framework for future investigations with respect to copy number control of pCU1.

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