FACTORS AFFECTING THE STABILITY OF *E.COLI* PLASMID VECTORS.

A thesis submitted for the Degree of
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by

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Dedicated to Mum and Dad

CONTENTS

Abbreviations

(i) Chemicals.

Ac - acetate
ATP - adenosine triphosphate
DNA - deoxyribonucleic acid
DTT - dithiothreitol
EDTA - ethylenediaminetetra-acetic acid (disodium salt)
EtBr - ethidium bromide
EtOH - ethanol
RNA - ribonucleic acid
SDS - sodium dodeecylsulphate
Tris - tris (hydroxymethyl) amino ethane

(ii) Antibiotics

Ap - Ampicillin
Stp - Streptomycin
Spc - Spectinomycin
Tc - Tetracycline

(iii) Phenotypes

X^r - resistance to X
X^s - sensitivity to X
oriV - replication origin
bla - B-lactamase
1^m - monomer
2^m - dimer
3^m - trimer

(iv) Measurements.

mA - milliamps (10^{-3} A)
bp - base pair
cpm - counts per minute
kb - kilo base pair (10^3bp)

kD - kilo Dalton

^{o}C - degrees centigrade

g - centrifugal force equal to gravitational acceleration

g - gramme

mg - milligramme (10^{-3} g)

ug - microgramme (10^{-6} g)

ng - nanogramme (10^{-9} g)

l - litre

ml - millilitre (10^{-3} l)

ul - microlitre (10^{-6} l)

M - Molar (moles per litre)

mM - millimolar

pH - acidity [negative $\log_{10}$ (Molar concentration H^{+} ions)]

V - volts

(v) Miscellaneous.

ln - natural logarithm

oc - open circular plasmid

cc - covalently closed circular plasmid

ds - double stranded DNA

UV - ultra violet light

fig - figure

B - beta

pfu - plaque forming units

moi - multiplicity of infection

a.a - amino acid(s)

PolI - DNA polymerase I

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Summary

Unlike natural plasmids, engineered plasmid vectors in general are not stably maintained in cell populations. This poses a great problem for industry where large scale fermentation of plasmid containing cells requires vast quantities of the appropriate antibiotics in order to select against plasmid-free segregants and therefore maximise product yield. Industrial applications apart, plasmid stability mechanisms constitute an interesting area of scientific research.

Analysis of the stability properties of pAT153 and pBR322 multimers revealed that they were unstable in the AB1157 derivative strain DS903. Moreover, as the multimeric state of these plasmids increased, both copy number and stability progressively decreased. This implied that an origin-counting mechanism probably operates for ColE1-like plasmids and that partitioning at cell division is random. The probability of plasmid-free segregants arising from cells containing multimeric plasids was minimal on the basis of their calculated copy numbers. Therefore, several factors which could explain the instability of these plasmids were discussed, these include plasmid sequestration, copy number variance and competition between plasmid-free and plasmid-containing cells.

Cloning the *par* region of pSC101 into multimeric plasmids failed to increase their stability although it is likely that *par* can operate in the monomeric forms of these plasmids. From this, it was concluded that *par* cannot function in all plasmids and its location within the molecule may be important to its activity.

Cloning the *rom* gene into the high copy number vector pUC8 reduced its copy number 6 fold but dramatically increased its stability. It is likely that cells possessing pUC8 (87 copies/genome equivalent) are at a competitive disadvantage to plasmid-free cells due to the metabolic burden imposed on them by pUC8. The stability of the *rom*$^+$ pUC8 plasmid derivative (14 copies/genome equivalent) can be attributed to a decreased growth differential between p$^-$ and p$^+$ cells, while the presence of the *rom* gene may lessen the probability of plasmid-free segregants arising by reducing copy number variance.

CHAPTER 1

INTRODUCTION

1.1 General Aspects of Plasmids.

Plasmids are extrachromosomal DNA elements which have been found in virtually all bacterial species. They encode a wide range of dispensible functions not normally encoded by the bacterial chromosome such as resistance to antibiotics, metal ions and organo-metallic compounds, enabling bacteria to exploit a diverse range of habitats. The ability of some plasmids to confer pathogenicity and antibiotic resistance on bacteria has resulted in plasmids becoming of central importance in clinical microbiology.

In addition to coding for accessory genetic traits, plasmids encode the determinants necessary for their vegetative replication, stable maintenance, control of copy number as well as conjugal transfer between bacteria (For reviews see Scott 1984; Willetts and Wilkins 1984). Plasmids can be conveniently classified into two categories on the basis of their replication behaviour. Large plasmids (>30kb) are generally present at 1-2 copies per cell and are termed as low copy number plasmids. Multicopy plasmids however, are usually small (<15kb), present in greater than 15 copies per cell.

The small size and high copy number of many plasmids, coupled to the ease with which they can be manipulated *in vitro*, led to their exploitation as tools in molecular biology and use in industry for cloning purposes. With the advent of gene cloning, much emphasis was placed upon biological containment considerations, provoking the construction of "disabled" plasmid vectors deleted for horizontal transfer functions, *mob* and *bom*, a region required *in cis* for mobilization by a conjugal plasmid. Very often these vectors unlike their naturally occurring parent plasmids, are not stably maintained in host cells. This is a major problem facing industry which often has to use vast quantities of expensive antibiotics in order to obtain maximum yields of cloned product.

1.2 Plasmid Maintenance

1.2.1 Replication General Considerations

In order that plasmids are stably maintained within bacterial cells, they must replicate at least once before cell division and be accurately partitioned to ensure that each daughter cell receives at least one plasmid copy at cell division.

The observation that different plasmids are maintained within cells at a particular copy number led to the idea that they controlled their own vegetative replication. Indeed, the first evidence to support this came from the isolation of copy mutants having a higher steady state copy number than the wild-type parent, which could be transferred between bacteria and still retain their copy mutant phenotype (Nordstrom 1972).

All plasmids share a few basic similarities in their replication properties. Firstly, they require host gene products for their replication (Scott 1984), hence plasmid encoded replication functions are generally confined to a small region of the plasmid molecule (usually 1-3kb) termed the minimal or basic replicon. A minimal replicon can be defined as the smallest piece of DNA that replicates with the wild-type copy number (Nordstom 1984). This consists of one or more replication origins and in some cases, a replication initiator protein (*rep*), although the actual mechanism of initiator protein function is not yet understood. Lastly, replication control functions (*cop* genes) are also located within the minimal replicon.

(i). Replication Origins.

Initiation of DNA replication occurs at defined origins, usually identifiable as "bubbles" in electron micrographs of replicating DNA. Some plasmids, like F and R6K possess several replication origins. Little is known about the relative activities of the replication origins in F, however, all 3 origins of R6K are used with equal frequency (Kolter 1981). The precise location of DNA synthesis initiation is unknown for most plasmids, but has been demonstrated to occur in the extensively studied ColE1 plasmid at any of 3 consecutive base pairs (Tomizawa *et al* 1977).

(ii). Plasmid Encoded Initiators

Several plasmids (generally low copy number) encode their own replication initiator proteins located adjacent to the replication origin. Their exact function remains to be elucidated, however, for two plasmids F and R1, formation of initiator proteins is rate-limiting for replication (Sogaard-Anderson *et al* 1984; Light and Molin 1981) and for R1 the Rep protein is mainly *cis* acting though it can act *in trans* under certain conditions (Masai *at al* 1983).

(iii). Replication Inhibitors.

Three different types of plasmid replication inhibitors have been demonstrated (1) Small RNA molecules e.g. RNAI of ColE1, *copA* of R1 (Tomizawa *et al* 1981; Stougaard *et al* 1981) (2) Small proteins such as ColE1 *rop*/*rom* and R1 *copB* (Cesareni *et al* 1982; Molin *et al* 1981) and (3) a series of direct repeats of DNA e.g. F, pSC101 and P1 (Tolun and Helinski 1981; Churchward *et al* 1983; Chattoraj *et al* 1984a). Intensive investigation into the "anti-sense" RNA control system of ColE1 has revealed the nature of replication inhibition in this plasmid. This occurs via base-pairing between the replication pre-primer RNA and the "anti-sense" RNA, however, little is known about the replication control kinetics of this plasmid. The control system of plasmid R1 gradually responds to copy number deviations in a proportional manner (Nordstrom *et al* 1984), while the F factor exhibits "switch-on switch-off" replication kinetics (Tsutsui and Matsubara 1981).

Two main models were proposed to explain control of plasmid replication prior to the demonstration that plasmids were indeed negatively regulated: the Inhibitor Dilution model (Pritchard 1969) and the Autorepressor model (Sompayrac and Maaloe 1973). The Inhibitor Dilution Model was initially proposed to explain the observation that chromosomal replication initiation always occurred at a constant cellular mass/chromosome ratio (Donachie 1968) but can be extended to plasmid replication control also. Basically, Pritchard predicted the existence of an inhibitor which was either synthesised in a burst during replication, or synthesised constitutively and was unstable. Cell growth leads to an increase in cell volume and hence a progressive

dilution of the inhibitor substance, whereupon replication initiation occurs and more inhibitor is synthesised. Small fluctuations in inhibitor concentration are assumed to have a large effect on the probability of replication. This model can explain replication control in unit-copy plasmids such as F, but is considered unlikely to be a plausible model for replication control of multicopy plasmids (Pritchard 1978). Fluctuation in the timing of individual initiation events of multicopy plasmids, prevents the necessary sharp transition between low and high concentration of replication inhibitor, which is required for "switch on- switch-off" replication kinetics.

The second control model predicted the existence of a replication inhibitor which was not constitutively expressed but instead was autoregulated. So far only one plasmid, lambda dv has been demonstrated to have such a control system (Murotsu and Matsubara 1980) and although several plasmids possess autoregulated replication initiator proteins (see later), they do not control the expression of an operon involved in replication initiation as predicted by the "autorepressor" model.

The essential feature of any replication control system which can correct deviations from the average copy number is a negative-feedback loop. This criterion is fulfilled for some plasmids by anti-sense RNA control and for others by the titration of an essential plasmid encoded initiator protein which is often autoregulated (Scott 1984).

The speed with which an inhibitor acts to correct copy number deviations, will depend upon several factors including whether the inhibitor is constitutively expressed or subject to some form of control, the stability of the inhibitor and thirdly, the kinetics of interaction of the inhibitor with its target (Nordstom et al 1984). The observation that naturally occurring plasmids such as ColE1 are stably maintained despite the lack of a par function (Summers and Sherratt 1984), indicates that replication control in these plasmids must either be very precise and copy number deviations do not occur, or if they do, are efficiently corrected. This may not be so for plasmid vector constructs and is discussed later in this thesis.

Nordstrom et al (1980a) proposed two models for replication control of plasmids assuming that they are partitioned randomly at cell division. Model 1 predicts that there is a mechanism which titrates the number of plasmid origins in a cell and raises the copy number to 2n in all cells (n=the average copy number per non-dividing cell). Model 2 (+n model)

predicts that irrespective of the plasmid copy number, the control system always adds "n" copies. This second model seems to fit the available data for plasmid R1 (Nordstrom et al 1984). By studying shifts between two steady-states of R1 copy number (using nutritional shift-up experiments and hybrid plasmid constructs), the copy number of R1 was shown to slowly adjust to the pre-shift value and the overall total rate of replication in the high copy number state was identical to that normally found for R1 (Gustafsson and Nordstom 1980). This implied that R1 replication is controlled by a system which determines the frequency of replication initiation without measuring the actual copy number. This observation is in direct contrast to that observed for F where a 2 fold increase in copy number results in a total switch off of replication (Tsutsui and Matsubara 1981).

The known replication strategies for several plasmids are discussed below.

1.3 Replication of Low Copy Number Plasmids

(A) The F-Factor.

The F plasmid (94.5kb) is present at a copy number of only 1-2 per chromosome equivalent and is therefore a unit copy plasmid. It is therefore categorised as a "stringently" replicated plasmid. An understanding of its replication mechanism was facilitated by the demonstration that a 9kb EcoRI fragment called "mini-F", encoded all of the replication and stability deteminants required for autonomous replication, showing that all the genetic information required for F replication was clustered together to form the "minimal replicon" (Lovett and Helinski 1976). The mini-F replicon encodes two replication origins oriV and oriS. Replication has been shown to proceed bidirectionally from oriV, but unidirectionally from oriS (see Scott 1984) and although an oriV deleted mini-F plasmid can replicate solely from oriS, it is unknown whether both origins function in wild-type F. Of the 9 proteins encoded by mini-F, the 29kD E protein is thought to be an absolute requirement for F replication, since insertions into this region, totally abolish replication. Moreover, F plasmids deleted for the E gene can be complemented in trans by the E containing region of a wild-type F plasmid (Tolun and Helinski 1982). Furthermore, mutations

within the E gene result in increased copy number, indicating that protein E may be rate-limiting for replication (Seelke et al 1982). By studying translational fusions between the E gene and lacZ, it was demonstrated that protein E synthesis is probably autoregulated (Sogaard-Anderson et al 1984) Sequence analysis has shown that the operator region overlaps the E gene promoter and is composed of a 10bp inverted repeat which is similar to repeats found in adjacent regions on the molecule. Two clusters of 19bp directly repeated sequences, which are inverted with respect to one another, have been found in the mini-F plasmid (Murotsu et al 1981). One of these clusters contains 5 direct repeats and lies to the right of the E protein reading frame and the other containing 4 repeats to the left. These regions are termed the incC and incB loci respectively and have been shown to be directly involved in F incompatibility, which implicates them in regulation of plasmid replication (Tolun and Helinski 1981; Tsutsui et al 1983). Similar multiple direct repeats between 17-22 base pairs in length have been demonstrated in other low copy number plasmids like P1, pSC101, RK2, Rts1 and in one intermediate copy number plasmid R6K. Like mini-F, these repeats have been shown to express the incompatibility properties of the parent plasmids P1, R6K and RK2 (Filutowicz et al 1984).

Deletion of several repeats in the mini-F plasmid results in an increase in copy number which is proportional to the number of repeats removed (Kline and Trawick 1983). This observation led to the proposal of a model for F plasmid replication control, which will be discussed below.

(B) Plasmid P1

Due to the great similarity between the control strategies adopted by both F and P1 plasmids, it is worth mentioning the recent data which has become available concerning P1 replication control.

P1 like F is a large plasmid (90kb), and has a low copy number and exists as a prophage, when not undergoing the lytic cycle. The organization of the replication origin regions of both P1 and F have been shown to be extremely similar (Abeles et al 1984). Like F, P1 possesses 2 clusters of 19bp repeats, situated either side of the region encoding an essential initiator protein repA (32kD). repA, like protein

Figure 1.1

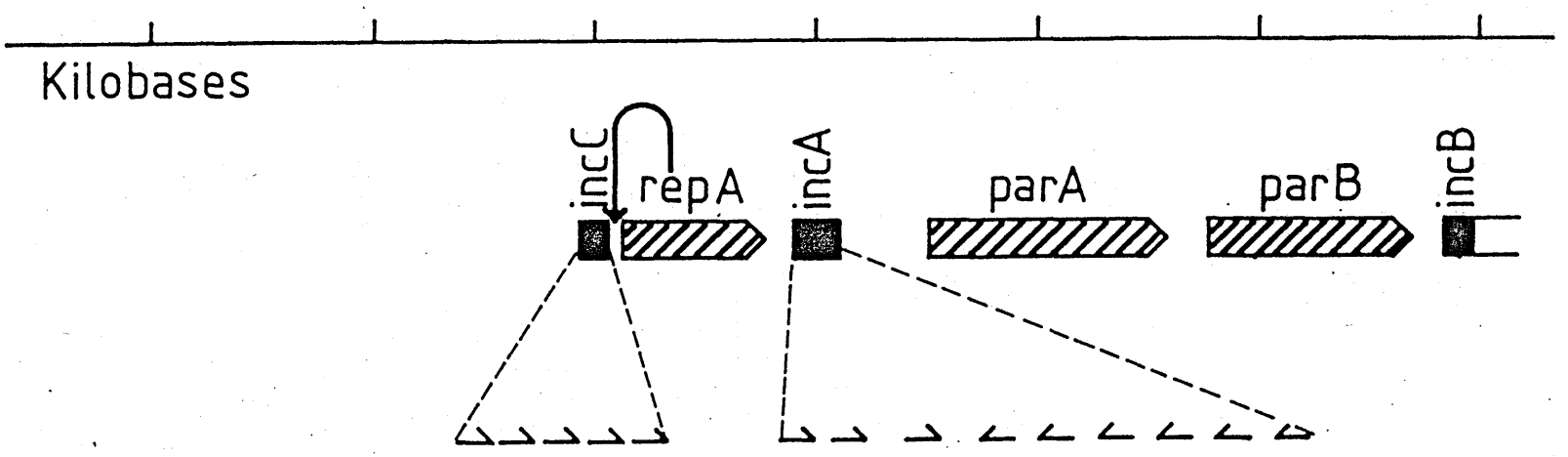


Figure 1.1 Replication Control Circuit of P1. There are 14 repeats of a 19bp sequence; 5 are located in incC within the replication origin and 9 in the second incompatibility locus incA downstream of the 32kD replication initiator protein RepA. The multiple repeated sequences which are bound by RepA, are proposed to titrate out the initiator protein, which prevents replication occurring until sufficient RepA protein has accumulated. Autoregulation of RepA is also involved in negative regulation of P1 copy number. Two proposed models to explain the antagonistic controlling systems are discussed in the text.

E, is subject to autoregulation (Chattoraj et al 1985). The replication origin contains 5 direct repeats and these constitute the incompatibility locus incC. Downstream of the repA coding region within incA, reside 9 further repeats, 6 of which are inverted with respect to incC (Fig 1.1). Deletion of the incA repeat sequences increases the copy number 6 fold, analogous to the result of deleting the incC locus of F. Furthermore, extra copies of the incA region of P1 effectively inhibit DNA replication of P1, while high levels of repA protein expressed from a compatible multicopy vector can overcome the inhibitory effects of the direct repeats (Chattoraj et al 1984). These data in addition to the demonstration that rep A protein binds to the repeated sequences (Chattoraj et al 1985), led to the formulation of a model to explain how replication is controlled in both F and P1. The multiple repeat sequences in F and P1 are thought to titrate out the initiator proteins E and repA, thereby maintaining the concentration of these factors below the critical threshhold level required for replication initiation. When these repeats become saturated, the concentration of repA rises and triggers replication. The additional binding sites created by replication then titrate out the initiator protein, whose concentration then falls, preventing further replication. The autoregulatory nature of both protein E and repA would tend to even out the fluctuations in initiator protein required to both trigger and prevent replication. To reconcile the two antagonistic control circuits, it is necessary to assume that there is a "lag" prior to the onset of autoregulation, such that transient increase and decrease in initiator protein act to effect or prevent replication before initiator synthesis is subject to autoregulation. The model proposes also that there is a hierarchy of affinities for the replication initiator such that the autoregulatory site has the highest affinity, followed by the copy number control repeats and the replication "trigger" site the lowest affinity (Chattoraj et al 1984).

A slight modification of this hypothesis has been suggested by Trawick and Kline (1985). They suggest that the replication initiator protein may differ from the autoregulator through modification, perhaps by oligomerization, which would explain how the two processes titration and autoregulation, do not cancel one another out.

It appears that multiple repeats and plasmid encoded initiator proteins are characteristic features of many low copy number plasmids,

Figure 1.2

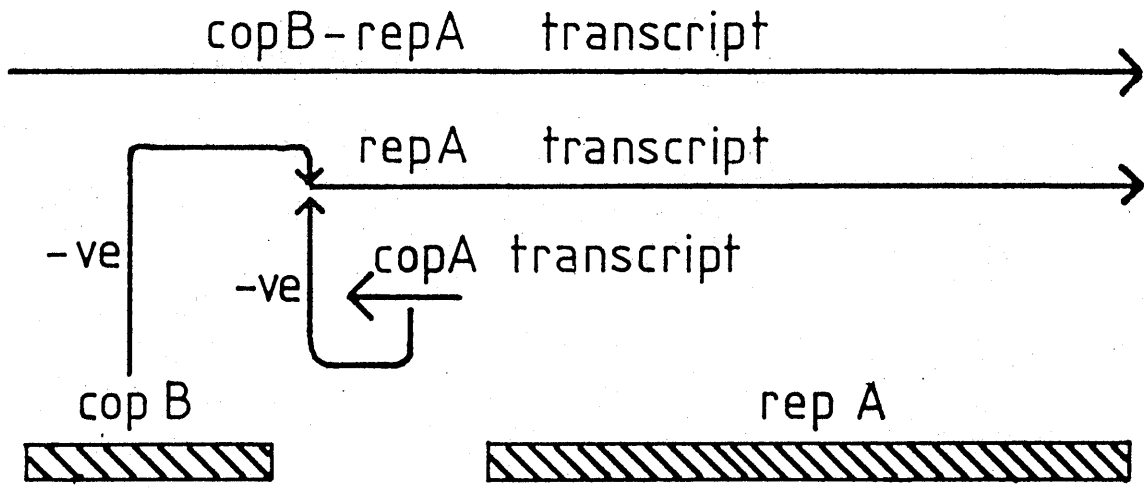


Figure 1.2 Plasmid R1 Replication Control Region. Transcription of repA in a wild-type R1 is primarily initiated from the copB promoter, so the longer transcript is the predominant species. copA RNA binds to the leader sequence of the repA message and prevents its translation, thereby inhibiting replication initiation. When the copy number drops below the normal level, the reduced level of CopB protein, results in the repA promoter being derepressed and therefore increased transcription of the repA gene occurs. Strong convergent transcription results in the production of a longer copA RNA molecule which is inactive, preventing post-transcriptional inhibition from occurring and replication inhibition proceeds.

since these have also been demonstrated in pSC101, Rts1 and RK2 (Vocke and Bastia 1983, 1985; Kamio *et al* 1984; Stalker *et al* 1981). The initiator proteins of F, P1 and pSC101 can be considered as both positive and negative effectors of replication, since they are absolutely required for replication, however, their autoregulatory nature suggests negative control. The multiple repeat sequences within and adjacent to the replication origin and the operator sequence of the initiator protein may result in cooperative binding of the initiator protein molecules in a manner similar to that discussed by Ptashne (1980). This kind of binding was considered to be one replication control mechanism which could account for the "switch on-switch off" type replication exhibited by F and probably other unit-copy plasmids. Presumably, small changes in the concentration of replication initiator dictate whether replication proceeds, resulting in an extremely precise replication control system which ensures that low copy number plasmids like F and P1 are not lost due to "sluggish" replication control. Other mechanisms which contribute to the maintenance of low copy number plasmids will be discussed later.

(C) R1 and Related Plamsids.

The low copy number plasmids R1, R6-5 and R100 which belong to the *inc*FII incompatibility group, exhibit a replication control mechanism which differs from both F and P1 and shares some basic similarity with the multicopy plasmid ColE1 (see later). Figure 1.2 schematically represents the replication control system of plasmid R1. 33kD protein designated *rep*A, is required for initiation of replication from the R1 origin (Light and Molin 1981). By constructing translational fusions between the *rep*A gene and *lac*Z, the R1 encoded *cop*A function was shown to inhibit the expression of the fusion protein (Light and Molin 1981). Inhibition acted at the post-transcriptional level (Molin and Light 1983). Further investigation determined that the *cop*A product was a small constitutively expressed, untranslated RNA molecule (80-90 nucleotides) with a high degree of secondary structure (Stougaard *et al* 1981). This molecule is proposed to bind the counter-transcript *rep*A message, disrupting the ribosome binding sites (Womble *et al* 1984). In addition, a second negative controlling element, the 86 residue *cop*B protein is involved although its precise role is uncertain. R1 plasmids

mutant in copB, exhibit an elevated copy number, which can be reduced in trans. However, excess copies of the copB gene do not affect the copy number of the wild-type R1 plasmid, which implies that there is no gene dosage effect on copy number (Riise et al 1982). Since copB, like copA is constitutively produced (Light and Molin 1982a) it was proposed that the level of copB protein expressed from wild-type R1 is sufficient to saturate its target site such that increased levels of the inhibitor do not result in increased inhibition. Indeed, Light and Molin (1982b) have demonstrated that the strong repA promoter is repressed to 10% of its maximum activity by copB, which explains the absence of gene dosage effect.

1.4 Replication Control of the High Copy Number Plasmid ColE1.

Plasmid ColE1 is a small (6.6kb) multicopy plasmid whose replication has been extensively studied both in vitro and in vivo (Staudenbauer 1978). Since ColE1 is the prototype plasmid of the vectors studied in this thesis, its replication properties will be thoroughly discussed (for reviews see Scott 1984; Cesareni and Banner 1985).

ColE1 has been shown to replicate unidirectionally from a fixed origin (Lovett et al 1974) by Cairns-type replication (Inselburg and Fuke 1971) and more recent studies have demonstrated that initiation of ColE1 DNA synthesis occurs at either one of 3 consecutive bases in the replication origin (Tomizawa et al 1977). Electron microscopic analysis of intermediates of ColE1 replication in the presence and absence of various dna gene products led to the proposal of a two-stage model for ColE1 replication (Staudenbauer 1978a). DNA Polymerase I extends the DNA chain from the RNA primer for 500 nucleotides, whereupon DNA Polymerase III continues to synthesise DNA on both strands. This explains why ColE1, unlike many other plasmids has an absolute dependence on PolI for replication (Kingsbury and Helinski 1973).

The observation that ColE1 plasmids could replicate for 10-15 hours after the addition of chloramphenicol, an inhibitor of protein synthesis, demonstrated that ColE1 does not require any plasmid encoded proteins for replication (Clewell 1972). Moreover, since bacterial replication cannot continue, the host proteins necessary for ColE1 replication must be stable.

ColE1 replication is however sensitive to Rifampicin, an RNA

Figure 1.3

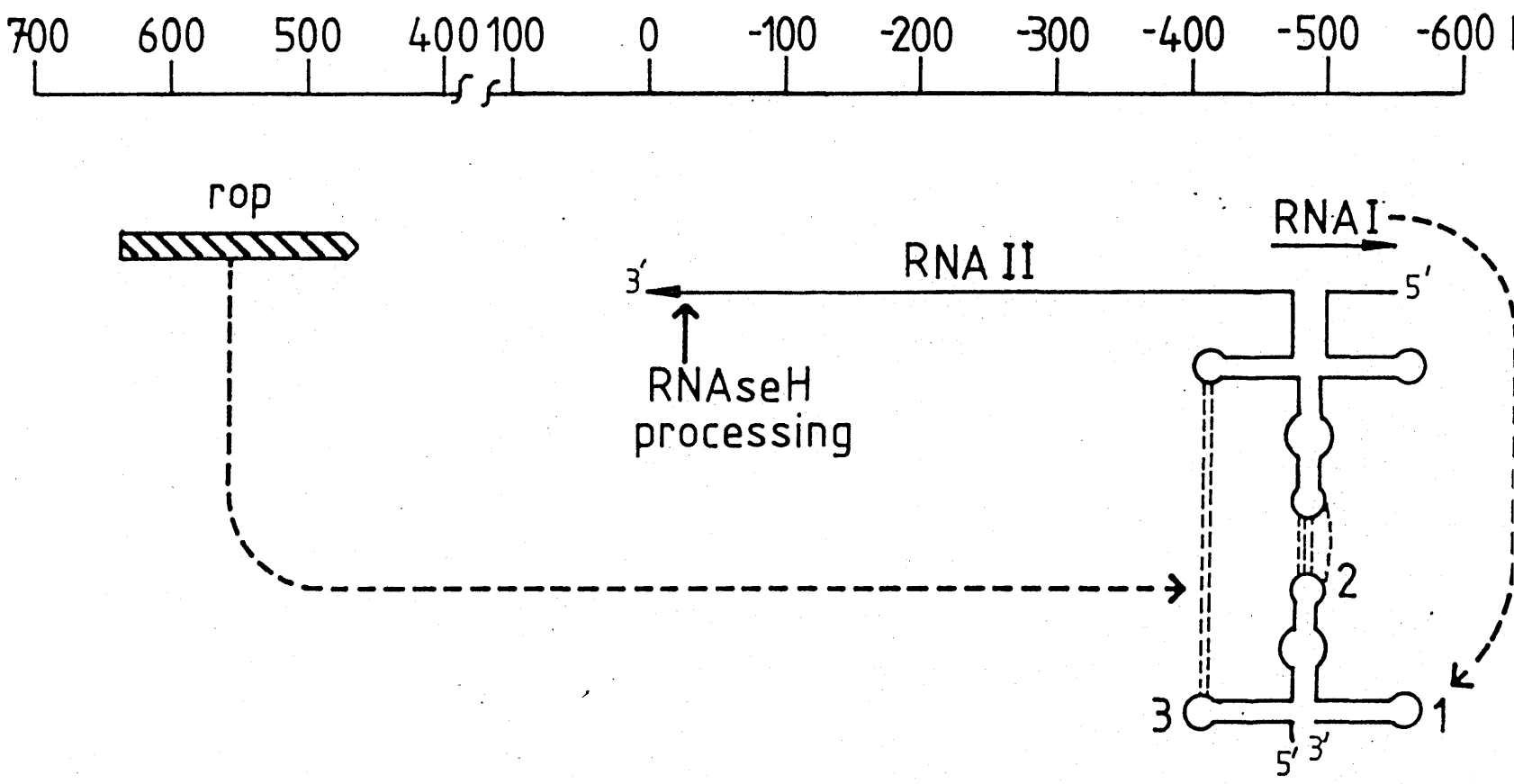


Figure 1.3 Replication Control Region of ColE1. Interaction between the complementary RNAI and RNAII molecules prevents processing of the pre-primer RNA (RNAII) by RNAase H to form the active replication primer. The 63 residue protein, Rom, catalyses the interaction between RNAI and RNAII, enhancing the negative replication control mechanism.

polymerase inhibitor, which first indicated that an RNA primer may be involved in the replication of this plasmid (Clewell 1972). The first direct evidence that ColE1 specified an RNA primer came from Itoh and Tomizawa (1980) who demonstrated *in vitro* that an RNA transcript initiating 555bp upstream of the defined origin, was cleaved by RNAase H indicating that an RNA-DNA hybrid was formed. After cleavage, DNA polymerase I added deoxyribonucleotides to the primer RNA (RNAII). Formation of the pre-primer RNA-DNA hybrid is negatively controlled by a small RNA molecule RNAI (108 nucleotides), which is encoded within the region specifying the pre-primer RNAII (Tomizawa *et al* 1981). In order for inhibition by the anti-sense RNA molecule to be effective, RNAI binding to the pre-primer RNAII must occur prior to the extension of RNAII to the replication origin (Tomizawa and Itoh 1981). Both RNAI and RNAII have the capacity to form three prominent stem-loop structures (Morita and Oka 1979) and the importance of secondary structure to RNAII processing has been demonstrated by replacing "G" residues with inosine which pairs weakly with "C" residues, disrupting secondary structure and inhibiting maturation of the primer.

Many elegant *in vitro* experiments have suggested a mechanism for the binding of RNAI to the RNAII pre-primer (Tomizawa 1984; see fig. 1.3). *inc* mutations within any of the three single standed loop regions, which do not alter the secondary structure of RNAI but affect the binding rate of RNAI to RNAII, demonstrated the importance of the loop regions to the binding process. Furthermore, the 5' end of RNAI is involved, since its removal greatly reduced the binding activity of RNAI to RNAII although once again the secondary structure of RNAI was not altered. From these data the "stepwise-pairing model" was proposed. Initially, RNAI and RNAII form stem-loop structures; the two molecules make transient contact, termed "kissing", at the single stranded loops. This contact facilitates pairing at the 5' end of RNAI, which then propagates along the entire length of RNAI/RNAII, forming stable binding.

In addition to RNAI, ColE1 specifies a second product called Rop or Rom, hereafter termed Rom (RNA one inhibition modulator) which is involved in replication control. The construction of a ColE1 deletion derivative exhibiting a higher copy number which could be reduced *in trans* by ColK, first suggested that another region of ColE1 was involved in copy number control (Twigg and Sherratt 1980). Later, Cesareni *et al* (1982) suggested that the *rom* gene located between the ColE1 replication

origin and mobility genes specified a 63 amino acid polypeptide (6.5kD). This protein has subsequently been purified (Som and Tomizawa 1983) and shown to inhibit transcription of the galK gene when it was fused to the primer promoter. Inhibition was strong when 135 bases of the primer region downstream of the promoter was fused to the galK gene but weak when only 52 bases were present, indicating that a region downstream of the first 52 bases was involved in the action of Rom protein.

RNAI has been shown to be an absolute requirement for Rom mediated inhibitory action both *in vitro* (Lacatena *et al* 1984) and *in vivo* (Cesareni *et al* 1984). *In vitro* studies on pMB1, a plasmid closely related to ColE1, have shown that Rom affects primer formation in two ways, firstly by causing transcription termination to occur at nucleotide 220 in 10%-20% of the primer transcripts and secondly, by increasing the ability of RNAI to inhibit RNAaseH processing of the primer.

Elegant *in vitro* experiments have demonstrated that Rom enhances the binding rate of RNAI to RNAII under physiological conditions (Tomizawa and Som 1984) by catalysing a stage which precedes the propagation of stable pairing between the two molecules. Furthermore, there is an inverse correlation between copy number and rate of binding of RNAI to RNAII and the enhancement caused by Rom protein is dependent on the particular RNA species i.e. wild-type or *inc* mutant forms of RNAI and RNAII. The highest binding rate was observed for the "wild-type" RNAI/RNAII species from ColE1, this was enhanced 2 fold by Rom. Enhancement of binding of the *inc* mutant forms was either greater or less than that observed for ColE1 RNAI/RNAII. These results were substantiated by *in vivo* data on the copy numbers of the corresponding rom^+/rom^- plasmids.

Thus, Rom enhances the inhibitory activity of RNAI however, the exact mechanism of replication inhibition is unknown. It is generally accepted that binding of RNAI to RNAII prevents processing of the pre-primer by RNAase H, but inhibition of expression of fusion proteins between galK or lacZ and part of the pre-primer argues to the contrary. It has been suggested that binding of RNAI makes RNAII more sensitive to degredation by ribonucleases, supporting the finding in another system involving binding of an RNA molecule to the complementary region of the 5' part of an mRNA (Coleman *et al* 1984).

Very recently, the structure of the Rom protein has been determined

and it has been shown to be a small rigid dimer of exact two-fold symmetry (Cesareni and Banner 1985). They have proposed that each subunit binds to either RNAI or RNAII and positions them correctly for the transient initial loop-loop interaction.

1.5 Stability Mechanisms

1.5.1 Partitioning.

In order that plasmids are stably maintained, in addition to controlled replication, they must be partitioned accurately at cell division so that each daughter cell receives at least one plasmid copy. The hereditary stability of low copy number plasmids such as F, pSC101 and R1 can only be explained by accurate partitioning occurring at cell division. Indeed, several low copy number plasmids have been shown to encode their own partition functions (Nordstrom et al 1980; Ogura and Hiraga 1983). For ColE1 and related plasmids however, there have been no substantiated reports of par-like functions and it has been suggested that active partitioning may not be critical for multicopy plasmids and random partitioning may suffice (Summers and Sherratt 1984).

Several models have been proposed for the partitioning of low copy number plasmids on the basis of curing kinetic data for ts replicons at non-permissive temperature. Equipartitioning results in each daughter cell receiving half of the number of plasmid copies at cell division (Novick et al 1975), while the Single-Site Model predicts that only the daughters of the last plasmid molecule replicated are strictly partitioned (Hashimoto-Gotoh and Ishii 1982). Supporting evidence for either model is however equivocal since curing kinetic data are difficult to interpret. Moreover, it has been suggested that such experiments cannot distinguish between the two models because of plasmid copy number variance between individual cells (Nordstom 1984). Despite uncertainty as to the actual partition mechanism(s), par functions have been demonstrated for several low copy number plasmids.

(A) pSC101

Plasmid pSC101, a derivative of the R plasmid SP219 (Cohen and Chang 1977) has a copy number of 5 per chromosome equivalent (Cabello et al

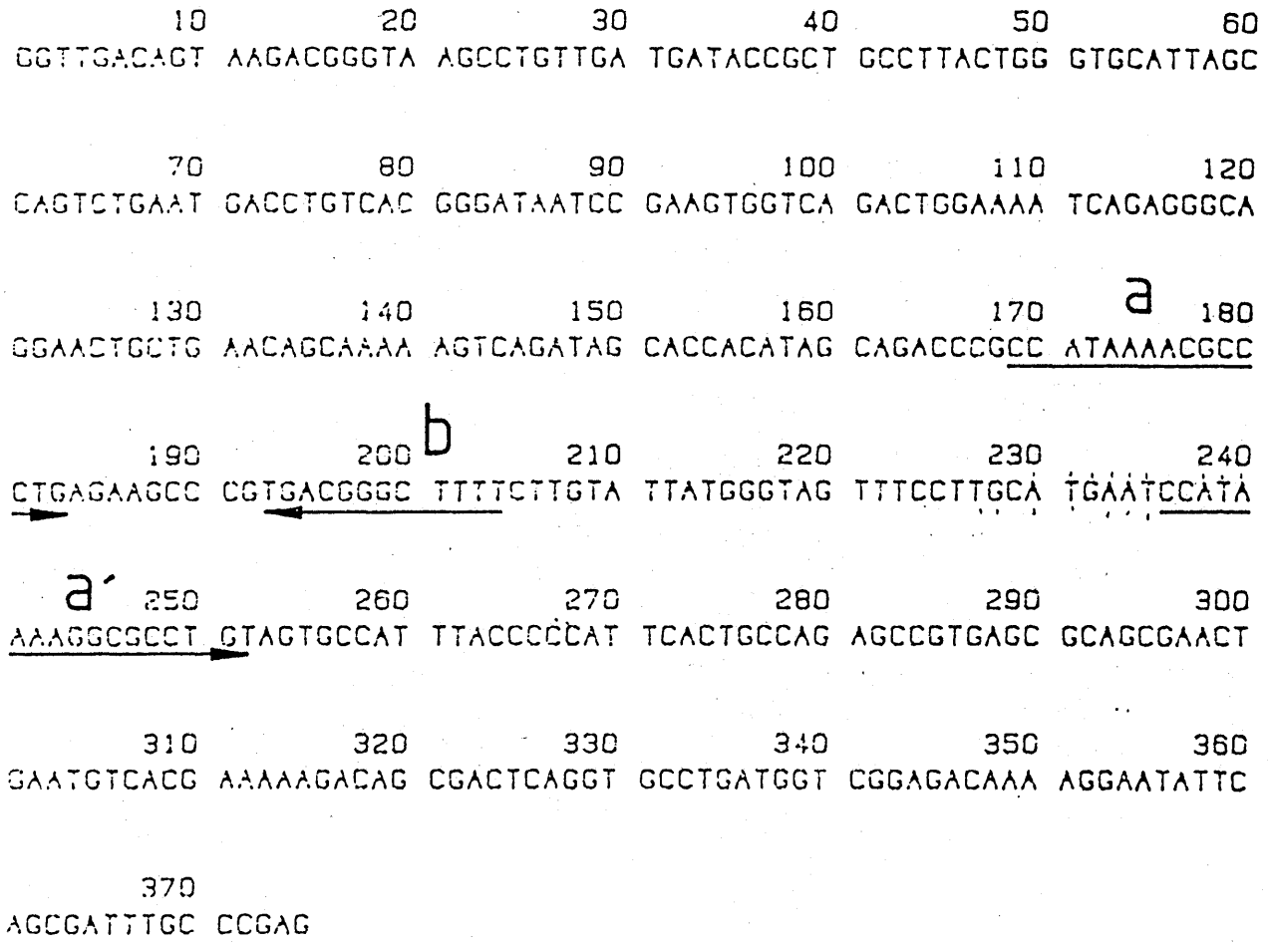


Figure 1.4 The pSC101 par Fragment. The "partition related segments" a, b and a' shown to be involved in par function are underlined.

1976). A 270bp region of pSC101 encodes the *par* locus, a *cis* acting stability region (Meacock and Cohen 1980). Its *cis* acting nature and lack of coding potential, indicate that it is most likely a site, which accomplishes active partitioning and may be analogous to the centromeres of eukaryotic chromosomes. In addition to stabilizing pSC101*par*$^-$ derivatives, it fully stabilizes an unrelated multicopy plasmid pACYC184. This indicated that *par* was not replicon specific, however, studies on the stability of various ColE1 derivative *par*$^+$ constructs, suggest that this may not be so (this thesis Chapter 4).

Fine deletion mapping analysis has delimited *par* to a 130bp region within the conveniently clonable 372bp EcoRI-AvaI fragment of pSC101 (see Fig 1.4, Kalla and Gustafsson 1984; Tucker *et al* 1984). Three discrete segments designated a, b and a' within this region are directly involved in the activity of *par* and therefore have been termed "partition related" segments. a and a' are direct repeats and 14/16bp are identical, while b is an inverted repeat of both, with 8/9 and 9/12 base matches respectively. The position and dyad symmetry of these segments are such that hairpin-loops could potentially form between the middle segment b with either a or a'.

Deletion analysis revealed that removal of any 2 of the 3 partition related segments resulted in a Par$^-$ phenotype, while removal of all 3 resulted in a "Super-par$^-$" phenotype, plasmids in this latter category being more unstable than the former category. Deletion of one segment produced a Cmp$^-$ phenotype although plasmids of this type were still stably maintained. The Cmp phenotype relates to the ability of a plasmid to transform a host strain already carrying an incompatible, wild-type pSC101 plasmid. These plasmids have a reduced transformation frequency which greatly exceeds that seen due to normal incompatibility, however, two Cmp$^-$ plasmids can stably co-exist and show normal incompatibility properties. The Cmp phenotype was demonstrated to be associated with the replication system of the plasmid by the stable co-existence of a Cmp$^-$ pSC101 derivative with a *par*$^+$ pACYC184 plasmid (Tucker *et al* 1984). It is possible, that the Cmp phenotype represents an intermediate stage in *par* function. Deletion of one segment may reduce the affinity of *par* for its binding site. If the replication enzymes are adjacent to this site, *par*$^+$ plasmids may be preferentially replicated, rather than Cmp$^-$ plasmids. The stable co-existence of two Cmp$^-$ plasmids indicates that both have an equal probability of being

replicated, while the stable co-existence of a Cmp$^-$ pSC101 plasmid with a *par*$^+$ pACYC184 implies that there may be different replication sites for unrelated plasmids. Furthermore, "Super-par$^-$" and Par$^-$ pSC101 derivatives with copy numbers equivalent to the wild-type, exhibit segregation frequencies which are several fold and slightly lower respectively than the calculated copy number of these plasmids. Also, *ts* pSC101 replicons at the non-permissive temperature for replication, are lost from cells at a rate consistent with the calculated copy number when they are *par*$^+$, but when they are Super-par$^-$, half of the cell population loses the plasmid after only one cell doubling, indicating that this latter group segregate as a single unit (Tucker *et al* 1984).

The above data imply that pSC101 plasmids possessing an intact *par* region can be counted as individual molecules and segregated evenly to daughter cells. Moreover, the Cmp$^-$ phenotype, suggests that the plasmids containing *par* regions deleted for one or more partition-related segments a, b or a' are at a replicative disadvantage in the presence of *par*$^+$ plasmids.

It has been suggested that *par* functions by binding to some host encoded component of the chromosome partitioning system. Gustafsson *et al* (1983) have demonstrated an association between the pSC101 *par* region and the outer membrane of *E.coli*.

(B) The F Factor

The partitioning system of the F-factor has been shown to reside within a 3kb segment, outwith but adjacent to the essential replication region. Trans-complementation analysis has revealed that 2 loci within this region, *sopA* and *sopB*, specify *trans*-acting functions, while the necessary *sopC* locus is *cis*-acting (Ogura and Hiraga 1983). *sopA* and *sopB* encode 41-44kD and 36-37kD proteins respectively (Wehlmann and Eichenlaub 1980; Komai *et al* 1982). The *sopB* protein in addition to two chromosomally encoded proteins of 75 and 33kD, have been demonstrated to bind to the region specifying the *cis*-acting locus *sopC* (Hayakawa *et al* 1985). These data prompted the proposal of a model for F partition. *sopC* is thought to bind to the cellular membrane via the *sopB*, 33kD and 75kD proteins. The role of the *sopA* protein is somewhat obscure, since it does not seem to bind to *sopC*, however, it may be involved in regulating expression of the *sopB* gene (Ogura and Hiraga 1983).

A 75kD host encoded protein has been shown to be involved in the binding of the chromosomal oriC region to the outer membrane of E.coli (Hendrickson et al 1982). It would be of interest to determine if this protein is identical to the 75kD protein which binds to the F-factor sopC region. If so, an attachment between sopC and the membrane could presumably be demonstrated.

In contrast to pSC101par, the F partitioning system seems to be involved in specifying incompatibility since the sopB region corresponds to incG and sopC to incD (Ogura and Hiraga 1983). This indicates that perhaps incompatibility between plasmids possessing the F partition system occurs due to competition for a limited number of partition sites on the bacterial membrane.

(C) P1.

Although P1 is a bacteriophage, it can also exist as a unit-copy plasmid and therefore requires to be accurately partitioned to prevent its loss from bacterial cells. The DNA region specifying P1 partition functions, is located adjacent to the replication origin and occupies a region of around 3kb. This region has recently been sequenced and the presence of open reading frames in addition to in vivo mutation data has produced a clear picture of the organization of the partition functions of this plasmid (Austin and Abeles 1984). The parA locus encodes a 44kD protein, which is thought to bind to the cis-acting incB site. An additional protein specified by parB (34kD) is encoded downstream of the parA gene and is probably co-transcribed from a promoter upstream of parA. Unfortunately, the 34kD polypeptide has not been demonstrated in maxicell profiles, which could be due to a low level of expression. However, B-gal fusion experiments have demonstrated that both parA and parB loci are involved in the downregulation of the parA parB operon, suugesting that parB is indeed real.

The cis-acting incB site, exerts strong incompatibility towards "compatible" plasmids which possess the cloned incB region and sequence analysis has demonstrated its highly A-T rich nature and the presence of a 20bp imperfect inverted repeat. The par proteins are presumed to bind to the incB site and the cell envelope to promote partition, however, at present like F partition, this is entirely speculative.

Several similarities exist between the F and P1 partitioning systems.

Firstly, both are located adjacent to and act independently from their respective replication origins and despite the functional similarity of the par regions they are plasmid specific and do not cross-react. Secondly, both encode two Par proteins which are subject to autoregulation and a cis-acting site (Austin and Wierbicki 1983). One or two components are incompatibility determinants. Finally, P1 and F partition systems can stabilize unrelated plasmids and neither location nor orientation affect par function.

It is possible that the elaboration of plasmid encoded par proteins is a feature specific to unit-copy plasmids in which absolute accuracy of partition is required. Presumably for the oligocopy pSC101 plasmid, this requirement is not quite as critical and host proteins may suffice.

(D) R1

Plasmid R1 exists at a copy number of 4-5 per cell and possesses a partitioning system which is less well characterized than the above systems. Deletion analysis followed by stability testing demonstrated that the par region was located some distance away from the replication origin, in contrast to the relationship between par and ori in F, P1 and pSC101 (Nordstrom et al 1980a). Since the par region is separated from the origin by the R-determinant, it is feasible that the two regions were once adjacent but became separated by the insertion of the resistance determinant. Initially, the partitioning function was thought to involve one locus, however, recent studies have demonstrated that two cis-acting loci, parA and parB are also involved (Gerdes et al 1985).

Both loci when cloned separately onto mini-R derivatives, stabilize the plasmid to a cerain degree but not fully. parA$^+$ parB$^+$ mini-R replicons are fully stable. parB can stabilize unrelated p15 derivative replicons more effectively than parA, suggesting that parA may be replicon specific requiring some other R1 function for its activity. As in the F and P1 partition systems, the R1 par genes were shown to exhibit weak incompatibility towards normally compatible plasmids carrying either parA or parB.

ClODF13, a multicopy replicon related to ColE1, has a copy number of 10 per cell and is reported to have a par function, parA which contributes weakly to the stability of this plasmid (Nijkamp et al 1984)

however, its mode of action is far from clear.

1.5.2 Plasmid Mediated Control of Cell Division

It has been known for some time that cell division is inhibited in cells which have not completed chromosome replication (Clark 1968). Evidence is now beginning to emerge which suggests that low copy plasmids also have the capacity to stall host cell division, until plasmid replication is completed.

The unit copy F factor, possesses two functions (CcdA and CcdB or LetA and LetD) which are involved in control of cell division. Ogura and Hiraga (1983a) demonstrated that an oriC plasmid containing a 700bp region of F, outwith the minimal replicon, in addition to F par, was more stably maintained than an oriC Fpar$^+$ plasmid. The letA and letD loci map within this 700bp region and are thought to be transcribed as a single unit. The isolation of amber mutations within both loci demonstrated that the 8kD and 11kD polypeptides predicted from sequence analysis are likely to be the products of the letA and letD genes respectively. When DNA replication of a ts F factor is prevented by growing cells at the non-permissive temperature, the cells continue to divide for about one generation and thereafter cell division ceases and non-septated filaments form. This does not occur with letA letD deleted F factors (Miki et al 1984a). Furthermore, inhibition of cell division also occurs when the letA letD region is cloned into unrelated plasmids such as pSC101 and pBR322 (Miki et al 1984a; Ogura and Hiraga 1983). By separately cloning the letA and letD genes, their individual functions were determined. LetD inhibits host cell division, while LetA suppresses the inhibitory activity of LetD and a model has been proposed to account for these findings. Before completion of F plasmid replication, LetD protein acts to inhibit host cell division. After completion of plasmid replication, the synthesis of LetA protein suppresses the inhibitory activity of LetD. At or after cell division, LetA is presumed to be inactivated or diluted out by an increase in cell mass, which once again allows inhibition of cell division by LetD to occur.

Thus in addition to the stability conferring par region, the F factor also encodes a second "fail-safe" mechanism which ensures that host cell division cannot proceed unless F plasmid replication has occurred. It

has been postulated that R1 also encodes a system which couples plasmid replication to cell division, however this is yet to be substantiated (Gerdes et al 1985).

1.5.3 Generalized Recombination: Formation of Plasmid Multimers.

Recently, it has been demonstrated that plasmid multimerization leads to plasmid instability (Summers and Sherratt 1984; Hakkaart et al 1984; Austin et al 1981; This work Chapter 3). There is evidence that the number of plasmid origins in a cell is kept constant by an origin-counting mechanism and formation of multimers results in a reduction of the number of segregating units available for segregation at cell division (Summers and Sherratt 1984; This thesis). Moreover, if there is an equal probability that replication initiation will occur at any plasmid origin in a cell, it is conceivable that any multimeric molecules which do arise may be preferentially replicated. Under conditions where homologous recombination is minimal, this will lead to the production of clones of cells possessing high levels of multimers, a reduced number of segregating units and which therefore will have a high probability of producing plasmid free segregants (James et al 1982; Summers and Sherratt 1984).

The major post-conjugational chromosomal recombination pathway in wild-type E.coli is the RecA RecBC pathway, while the RecA RecF pathway plays a minor role (Clark 1973). Intra-chromosomal recombination however, may involve the RecA RecF pathway to a greater extent (D.Sherratt pers. comm). Plasmidic recombination occurs mainly via the RecA RecF pathway and does not require functional recB recC gene products (Laban and Cohen 1981). In recombination proficient hosts, inter-molecular recombination between plasmids leads to the production of multimers; tandem repetitions of the monomeric unit which run as discreet plasmid DNA bands running higher than the monomer supercoil on agarose gels (Bedbrook and Ausubel 1976). Intra-molecular recombination results in the breakdown of plasmid multimers to monomers. Since mutations in recF, reduce plasmid recombination to around 3% of that seen in Rec^+ cells, $recF^-$ strains are therefore suitable for maintaining specific plasmid forms (Cohen and Laban 1983; Summers and Sherratt 1984).

A third pathway operating in E.coli is the RecE pathway. Many

E.coliK12 strains carry a prophage called Rac which encodes the recE gene product, exonuclease VIII. The sbcA mutation (suppressor of recBC) de-represses the synthesis of exonuclease VIII, and recBC sbcA host strains have been found to be hyper-recombinogenic for plasmids. In these strains, inter-plasmidic recombination is increased 2-5 fold in comparison to wild-type strains (Kaiser and Murray 1979; Laban and Cohen 1981).

AB1157 and its derivatives (predominantly used in this thesis), are deleted for Rac and consequently stimulation of plasmidic recombination by the RecE pathway cannot occur. One AB1157 derivative, JC8679 (recBC sbcA) acquired an intact copy of Rac during its construction via mating from an Hfr strain carrying the sbcA23 mutation into a recBC derivative of AB1157 (Kaiser and Murray 1980). It was frequently used in this thesis to facilitate the isolation of plasmid multimers,

1.5.4 Multimer Resolution Systems

The multicopy plasmid ColE1, unlike many related engineered plasmid vectors, rarely forms multimeric molecules in Rec^+ hosts and is stably maintained. This suggested that it may have a mechanism which resolves multimeric molecules to monomers thereby maximizing the number of segregating units. By analysing the extent of multimerization of a range of HaeII deleted ColE1 plasmids in the hyper-recombinogenic host (JC8679), the monomerizing "function" was located within the 1.9kb HaeIIB fragment of ColE1, between the mobility region and the colicin E1 structural gene (Summers and Sherratt 1984). Subsequent deletion analysis of this region isolated a minimal fragment of 280bp within which cer the ColE1 resolution function lay. This region is too small to encode a recombinase enzyme and extra ColE1 sequences are not required for cer function, since the 280bp region alone is sufficient to monomerize pUC8 plasmids. The process is independent of RecA, RecE and RecF pathways.

It has been suggested that the E.coli chromosome encodes a recombinase which effects site-specific recombination between directly repeated cer sequences on multimers. Indeed, chromosomal mutations have recently been isolated which lack this activity and multimeric cer containing plasmids can be maintained in these hosts (G. Stewart pers comm.). The site-specific nature of the recombination event was formally shown by

the production of a functional hybrid between cer and ckr, the analogous region of ColK, a close relative of ColE1, sharing more than 90% homology with ColE1 in this region (Summers et al in press 1985). Analysis of several hybrid sequences located the cross-over site to a 35bp region which contains palindromic symmetry. Unlike the directional specificity requirement of some site-specific recombinases such as Tn3 resolvases, which strongly prefers directly repeated res regions (Krasnow and Cozarrelli 1983) and Hin, Gin and Cin which prefer inverted sites (Plasterk et al 1983), both cer and ckr can cause deletion and inversion with directly and indirectly repeated sites respectively (Summers et al 1985 in press).

The closely related plasmid CloDF13 (10 copies/cell) possesses an analogous region to cer designated parB, which resolves multimeric molecules to monomers (Hakkaart et al 1984). This region is 69% homologous with the 150bp "core" region of ColE1 cer, but does not show the same palindromic symmetry in the cer cross over region (Summers et al 1985). Also unlike cer, parB is only required for stable maintainance of copy mutant CloDF13 plasmids. In wild-type plasmids this function is dispensible.

Computer searches of DNA sequence banks have demonstrated that pMB1, another close relative of ColE1, shows almost complete homology with the ColE1 cer region. It is possible therefore, that cer-like functions which maximise the number of segregating units may be a common feature of multicopy plasmids (Summers and Sherratt 1985).

Whereas in the multicopy situation, multimerization may lead to instability, dimerization of a unit copy plasmid will undoubtedly result in hereditary instability. A multimer resolution system has been demonstrated for the unit copy plasmid P1. P1 encodes a site loxP at which the recombinase Cre (35kD) acts to promote dimer breakdown and increase P1 stability (Sternberg and Hamilton 1981; Abremski and Hoess 1984; Austin et al 1981). Cre has a general DNA binding affinity, but binds loxP sites specifically with 20 fold increased binding efficiency (Abremski and Hoess 1984). The cross-over site in loxP was determined to lie within an 8bp region which separates two perfect 13bp inverted repeats (Hoess et al 1982) and deletion analysis revealed that a 39bp region containing the inverted repeat was sufficient for full function (Hoess et al 1984). The loxP site is dissimilar to the well characterised res and attP functional recombining sites of Tn3 and

lambda which are 100bp and 240bp respectively (Hsu *et al* 1980; Grindley *et al* 1982). The Tn3 *res* site comprises three resolvase binding sites and the cross-over site is asymmetrically located within the first site. The current hypothesis is that the remaining sites provide the required directionality for the resolution reaction (M.Boocock *et al* Biochem. Trans. in press), while the large lambda *att*P site may be required for recognition by several different protein molecules concerned with the decision of excision or integration.

The aim of this thesis was to determine which factors affect the hereditary stability of several ColE1 derivative recombinant plasmid vectors. In particular, the contribution to stability from plasmid multimerization and copy number were studied. Additionally, the stabilizing effect of the *par* locus from pSC101 on these replicons was examined.

CHAPTER 2

MATERIALS AND METHODS

Table 2.1 Bacterial Strains

Name	Relevant Markers	Source
DS903	Stp^r *recF thr leu thi pro his arg*	D.Sherratt
JC8679	*recBCsbcA* AB1157 deriv.	A.J.Clark
△M15	△[*lac pro*] Ø80d*lacZ* M15	U.Ruther
PO810	*polA12*$_{ts}$ Stp^r Spc^r fad::Tn10 Tc^r	P.Oliver
CT001	as DS903 but *polA12*$_{ts}$ fad::Tn10	Chapter 6
DS835	Spc^r Stp^s	D.Sherratt
CJ100	Spc^r Stp^s DS903 derivative by transduction from DS835	Chapter 6

Table 2.2 Plasmids.

Name	Description	Phenotype	Size (kb)	Source or Reference
pAT153	*rom*⁻ *bom*⁻ deletion derivative of pBR322	Ap^r Tc^r	3.672	Twigg and Sherratt (1980)
pBR322	vector derived from pMB1	Ap^r Tc^r	4.363	Sutcliffe (1978)
pCJ101	pAT153 deleted for the Tc^r region + the 372bp *par* fragment	Ap^r	2.6	Chapter 4
pUC8	vector derived from pBR322	Ap^r	2.67	Vieira and Messing 1982
pCJ112	pUC8 + 309bp *rom* fragment	Ap^r Rom^+	2.98	Chapter 6
pCJ113	" " " " oppos. orient.	Ap^r Rom^+	2.98	"
pCJ103	pAT153 + 53bp Sau3A fragment from ColE1	Ap^r Tc^s	3.72	Chapter 3
pCJ104	pAT153 + 81bp PstI fragment from ColE1	Ap^s Tc^r	3.75	Chapter 3
pCJ105	pBR322 + 81bp PstI fragment from ColE1	Ap^s Tc^r	4.4	Chapter 3
pCJ106	pBR322 with 15bp deletion around BamHI	Ap^r Tc^s	4.3	Chapter 3
pCJ108	dimer constructed from pCJ104 and pCJ103	1Ap^r Tc^r	7.47	Chapter 3
pCJ109	trimer " " " "	1Ap^r Tc^r	11.2	Chapter 3
pCJ110	dimer " " pCJ105 and pCJ106	1Ap^r Tc^r	8.7	Chapter 3
pCJ111	trimer " " " "	1Ap^r Tc^r	13.1	Chapter 3
pLB04	pUC9 + 1.9kb HaeII *cer* fragment of ColE1	Ap^r Cer^+	4.8	Lesley Brown this lab
ColE1	naturally occurring	$Ce1^+$ $Ie1^+$	6.6	D.Sherratt
pKS451	pUC9 + 377bp *cer* fragment from ColE1	Ap^r Cer^+	3.1	D.Summers
λB31rom	λ*dv* + *bom*, *rom* and *mob*1 of ColE1	Cm^r Rom^+	6.0	C.Boyd

Table 2.2 Plasmids / continued

Name	Description	Phenotype	Size (kb)	Source or Reference
pCJ107	pKS501 + 409 HaeIII/SmaI fragment from pCJ101 containing *par*	Ap^r Par^+ Cer^+	5.8	Chapter 4
pKS501	pUC9 + 2.9kb PstI fragment from ColE1::Tn1	Ap^r Cer^+	5.4	D.Summers
pKS511	pLB04 + 703bp PstI ColE1::Tn1 fragment	Ap^r Cer^+	5.2	D.Lee
pCJ114	1st amplified form of pCJ107, 2 origins	As pCJ107	7.3	Chapter 4
pCJ115	2nd " " " " 3 "	" "	8.5	Chapter 4
pCJ118	pKS511 + EcoRI linkered *par* fragment	$Ap^rCer^+Par^+$	5.6	Chapter 4
pCJ119	1st amplified form of pCJ118, 2 *par*	As pCJ118	7.9	Chapter 4
pCJ120	2nd " " " " 3 *par*	" "	10.1	Chapter 4
pCJ121	pBR322 + 5628bp Lambda DNA insert.	Ap^r Tc^s	10.0	Chapter 3
pCJ122	pUC8 + 411bp HaeIII *par* fragment	Ap^r	3.0	Chapter 4
pCJ123	pUC9 + HindIII/EcoRI polylinker of pCJ122, *par* is in oppos. orientation	Ap^r Par^+	3.0	Chapter 4
pST2	pAT153 + EcoRI linkered *par* fragment	$Ap^rTc^rPar^+$	4.0	W.Tacon
pWX9	pBR322 + 372bp *par* containing fragment Tc^r region removed	Ap^r Par^+	3.1	P.Derbyshire

Notes to Table 2.2 1Ap^r = one functional *bla* gene.

Table 2.3.

CHEMICAL	SOURCE
General chemicals and organic compounds	BDH, Hopkins and Williams, Koch-Light Laboratories, May and Baker
Media	Difco, Oxoid
Biochemicals	Sigma
Antibiotics	Sigma
Agarose	BRL
Restriction enzymes	BRL
X-gal	BRL
Sephadex G-50	Pharmacia Fine Chemicals
SDS	Serva
Atomlight Scintillant	NEN
Benzyl penicillin	Glaxo
Methyl-^{3}H thymidine	Amersham

2.1 Bacterial Strains

The bacterial strains used were all derivatives of *Escherichia coli* K-12 and are listed in table 2.1. Genotype and phenotype symbols are those recommended by Bachman *et al* (1976) and Novick *et al* (1976).

2.2 Plasmids

The plasmids used in this study are listed in table 2.2. Nomenclature follows Novick *et al* (1976).

2.3 Culture Media

L-Broth: 10g tryptone, 5g yeast extract, 5g NaCl, 1g glucose, 20mg thymine, made up to 1 litre in distilled water and adjusted to pH7.0 with NaOH.

Nutrient Agar: 25g Oxoid nutrient broth No.2, 12.5g agar, made up to 1 litre in distilled water.

Minimal Agar: 7g K_2HPO_4, 2g KH_2PO_4, 4g NH_4SO_4, 0.25g trisodium citrate, 0.1g $MgSO_4.7H_2O$, 17.5g agar, made up to 1 litre in distilled water.

Isosensitest Agar: 23.4g isosensitest broth, 12.5g agar made up to 1 litre in distilled water.

Davis-Mingioli Salts (x4): 28g K_2HPO_4, 8g KH_2PO_4, 4g $(NH_4)_2SO_4$ 1g sodium citrate, 0.4g $MgSO_4.7H_2O$, made up to 1 litre with distilled water.

D/M Minimal Medium: 25ml D/M salts, 5ml 20% casamino acids, 250ul 20% glucose, 0.5ml vitamin B1 (1mg/ml), made up to 100ml with water.

M9 Salts (x10): 6g Na_2HPO_4, 3g KH_2PO_4, 0.5g NaCl, 1g NH_4Cl in 1 litre distilled water.

M9 Minimal Medium: 10ml M9 salts, 2ml glucose(20% w/v), 0.1ml 1M $MgCl_2$, 0.1ml 100mM $CaCl_2$, 0.1 ml (1mg/ml) vitamin B1, 2.5ml (20%) casamino acids, made up to 100ml with distilled water.

Phage Buffer: 7g Na_2HPO_4, 3g KH_2PO_4, 5g NaCl, 0.25g $MgSO_4$, 15mg $CaCl_2.2H_2O$, 1ml 1% gelatin made up to 1 litre in distilled water.

P1 Adsorption Medium: 10ml L-broth, 1ml 1M $CaCl_2$, 89ml dH_2O.

R-Broth: 10g Bacto tryptone, 1g Bacto yeast extract, 8g NaCl. After autoclaving add 2ml 1M $CaCl_2$ and 5ml (20%) glucose.

R-Broth Top Agar: As R-broth but with addition of 8g Difco minimal agar.

Continuous Culture Medium: 20g casamino acids, 108.8g KH_2PO_4, 26.44g $(NH_4)_2SO_4$, 100ml chelated metals, pH'd with 8g NaOH pellets, made up to 19.9 litres and autoclaved (pH7). A 100ml solution of 1M glucose, 0.1M $MgSO_4$, 100mg vitamin B1 was added to this after autoclaving separately.

Chelated Metals: 50g nitrilotriacetic acid, 125ml 10N NaOH, 500ml dH_2O pH'd to 7.0 with HCl. The following trace elements were then added; 1.1g $FeSO_4.7H_2O$, 50mg $Na_2MoO_4.2H_2O$, 50mg $ZnSO_4.7H_2O$, 25mg $CuSO_4.6H_2O$, 25mg $CoCl_2$. Made up to 1 litre.

Supplements: When required, supplements were added to minimal media at the following concentrations:

glucose 2mg/ml	amino acids 40ug/ml
thymine 50ug/ml	thiamine vitamin B1 20ug/ml
casamino acids 1%	

2.4 Sterilization

All growth media were sterilized by autoclaving at 120°C for 15 minutes; supplements, gelatin solution and buffer solutions at 108°C for 10 minutes and $CaCl_2$ at 114°C for 10 minutes.

2.5 Buffer Solutions

2.5(a). Electrophoresis

10x TBE Buffer pH8.3: 109g Tris, 55g Boric Acid, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water; pH is 8.3.

10x E Buffer: 48.4g Tris, 16.4g NaAc, 3.6g $Na_2EDTA.2H_2O$, made up to 1

litre in distilled water, pH adjusted to 8.2 with acetic acid.

Single Colony Gel Buffer: 2% Ficoll, 1% SDS, 0.01% Bromophenol Blue, 0.01% Orange G in buffer E.

Horizontal Agarose Gel Loading Buffer: 10mM Tris.HCl (pH 7.5); 20mM EDTA; 10% Glycerol; 0.01% Bromophenol Blue; 2mg/ml agarose. After melting agarose mixture at 100°C, allow to set and disrupt the slurry to a smooth emulsion by passage through a hypodermic needle.

Final Sample Buffer: 10% Ficoll, 0.5% SDS, 0.06% Bromophenol Blue, 0.06% Orange G in 1xTBE.

2.5(b) DNA Manipulation

Restriction Buffers:

10x Low Salt: 100mM Tris-HCl pH 7.5, 100mM $MgSO_4$, 10mM dithiothreitol; store at -20°C.

10x Medium Salt: 500mM NaCl, 100mM Tris-HCl pH 7.5, 100mM $MgSO_4$, 10mM dithiothreitol; store at -20°C.

10x High Salt: 1M NaCl, 500mM Tris-HCl pH 7.5, 100mM $MgSO_4$, 10mM dithiothreitol; store at -20°C.

10x SmaI Buffer: 200mM KCl, 100mM Tris-HCl (pH 8.0), 100mM $MgCl_2$, 10mM dithiothreitol; store at -20°C.

10x Ligation Buffer: 660mM Tris-HCl pH 7.6, 100mM $MgCl_2$, 10mM EDTA, 100mM dithiothreitol; store at -20°C.

ATP For Ligations (4mM): 4mM ATP in 4mM Tris-HCl pH7.5

TE Buffer: 10mM Tris-HCl, 1mM EDTA; pH 7.6.

2.5(c). DNA Hybridization

10x Nick Translation Buffer: 500mM Tris-HCl pH7.2, 100mM $MgSO_4$, 1mM dithiothreitol, 500 ug/ml Bovine Serum Albumin; store at -20°C.

Denhardt's Solution: 0.2 mg/ml Bovine Serum Albumin, 0.2 mg/ml Ficoll-400, 0.2 mg/ml Polyvinylpyrolidone; store at -20°C.

20x SSC: 3M NaCl, 300mM trisodium citrate pH 7.0.

20x SSPE: 3.6M NaCl, 200mM NaH_2PO_4, 20mM Na_2EDTA pH 7.4.

Gel Soak I: 1.5M NaCl, 0.5M NaOH

Gel Soak II: 1M Tris, 1.5M NaCl pH8.0

Pre-hybridizing Solution: 5x Denhardt's solution, 5x SSPE, 0.5% (w/v) SDS, 500ug/ml denatured salmon sperm DNA.

Hybridization Solution: As above but ^{32}p-labelled probe added.

Wash Buffer: 5mM NaH_2PO_4, 1mM Na_2EDTA, 0.2% SDS; pH7.0

2.5(d). DNA Preparation Solutions.

Birnboim Doly I: 50 mM Glucose, 25mM Tris-HCl pH 8.0, 10 mM EDTA; add lysozyme to 1 mg/ml immediately before use.

Birnboim Doly II: 0.2M NaOH, 1% SDS; freshly made.

Birnboim Doly III: 5M KAc pH 4.8; mix equal volumes of 3M CH_3COOK and 2M CH_3COOH; pH should be 4.8.

Sucrose Solution: 25% Sucrose, 50mM Tris pH 8.0.

Lytic Mix Solution: 1% Triton X-100, 50mM Tris, 60mM EDTA; pH 8.0.

ZIPPY Buffer: 50mM Tris-HCl pH 8.0, 50mM EDTA, 8% Sucrose, 5% Triton X100; add 50ul of 10 mg/ml lysozyme immediately before use.

Tautz and Renz I: 300mM NaAc pH 7.0, 1mM EDTA.

Tautz and Renz II: 1M $MgCl_2$, 10% Acetic acid.

Tautz and Renz III: 100mM NaAc pH 6.0, 70% EtOH.

2.6 Antibiotics

The antibiotic concentrations used throughout this work in both liquid and plate selection were as follows:

Name	Source of Resistance	Selective conc.	Stock Soln
Ampicillin(Ap)	plasmid	50ug/ml	5mg/ml(dH_2O).
Tetracycline(Tc)	plasmid	10ug/ml	1mg/ml(0.1MNaOH).
Streptomycin(Str)	chromosome	50ug/ml	5mg/ml(dH_2O).
Spectinomycin(Spc)	chromosome	50ug/ml	5mg/ml(dH_2O).
Cephaloridine	plasmid	as indicated	100mg/ml(dH_2O).
Kanamycin(Km)	P1	50ug/ml	5mg/ml(dH_2O).

All stock solutions were stored at 4°C and when necessary added to molten agar pre-cooled to 50°C.

2.7 Indicators: X-gal (5-Bromo-4chloro-3indolyl-B-Dgalactoside) was stored at a concentration of 20mg/ml in dimethyl-formamide at -20°C and added to L-agar plates at a final concentration of 20ug/ml. This gratuitous substrate was used in conjunction with the M15 host strain and pUC vectors. When the polylinker regions of pUC plasmids are not interrupted by the presence of cloned fragments, complementation between the plasmid-encoded partial B-galactosidase polypeptide and the partially deleted chromosomal locus, produces a functional polypeptide. This hydrolyses the colourless X-gal compound and results in blue colouration of colonies possessing pUC vectors without cloned inserts. Consequently this provides a convenient screening for plasmids possessing cloned inserts in the polylinker region since these colonies

are white.

2.8 Growth Conditions

Liquid cultures for transformation of plasmid DNA preparations were routinely grown in L broth at 37°C with vigorous shaking. Stationary phase overnight cultures were grown from small inocula in 2.5 ml L broth without shaking at 37°C.

Growth on plates was on L-agar, isosensitest or minimal medium plus supplements. Antibiotics were added as required. Plates contained 25 ml agar solution and were incubated for approximately 15 hours at 37°C. All dilutions for plating out cells was carried out in phage buffer.

Bacterial strains were stored in 50% L broth, 40% glycerol at -20°C. Inocula from these were applied to selective plates and after growth at 37°C single colonies were selected for use.

2.9 In Vivo Techniques

Transformation of E.coli: Plasmid and phage DNA can be introduced into E.coli cells by a process known as transformation. E.coli is not naturally competent to take up DNA from the environment, but can be made to do so by treatment with $CaCl_2$. This makes a proportion of the cell population competent. Sterile plastic lab-ware was used throughout because competent cells are easily lysed by detergents. A fresh stationary overnight culture of cells was diluted 1/100 in L broth and grown at 37°C to mid log phase (1x 10^8 cells/ml) requiring approximately 90 minutes. The culture was rapidly chilled and pelleted by centrifugation at 5K for 2 minutes at 0°C (3020 g). The pellet was resuspended in 10 ml 50mM cold $CaCl_2$ and repelleted. The cells were resuspended in 5 ml cold $CaCl_2$ and incubated at 0°C for 20 minutes. A final pelleting and resuspension in 1 ml cold $CaCl_2$ was then carried out. 200 ul aliquots of cell suspension were added to DNA solutions and incubated at 42°C for 2 minutes; ligations were diluted 1/10 and 1/100 with $CaCl_2$. After heat shocking, the cells were kept at 0°C for an hour during this period DNA is taken up. For transformation to ampicillin resistance no expression time was necessary. When selecting for tetracycline, streptomycin and spectinomycin transformants, 1 ml of L broth was added to the cell DNA suspension and incubated at 37°C for 90

minutes.

B-Lactamase Plate Assay: This assay was developed by Boyko and Ganschow (1982). It is based upon the removal of iodine from bacterial colonies and from a starch-iodine complex by penicilloic acid which is formed when penicillin is cleaved by B-Lactamase.

The stock iodine solution consists of 200g KI and 40.6g I_2 made up to 1 litre in dH_2O and should be kept in a light proof reagent bottle. 20ml of Benzyl penicillin (3g/100ml stored at 4°C) and 3ml of the iodine solution are mixed together and used to flood the test plates. After the solution has uniformly stained the plates a bluish-black colour (10-15 secs), the mixture is discarded and the plates allowed to drain.

Initially, all colonies are dark-yellowish brown, however, p^+ colonies soon begin to turn white and the stained agar surrounding the colony begins to clear, until a "halo" around the colony is formed. This "halo" increases in size with time. In contrast, plasmid free colonies remain brown in colour and no localized clearing occurs. After 15 minutes, there is a general clearing of the media.

To obtain optimum results with this technique, it is advisable to have less than 200-300 colonies per plate, otherwise clearing around one colony may also affect adjacent colonies. Finally, iso-sensitest agar is used in preference to L-agar because it contains soluble starch which is required for the blue-black colouration. L-agar may be used when 2% (w/v) soluble starch is added but was not used in this work.

Stability Testing: A single colony from a selective plate was used to inoculate2.5ml L-broth which contained the appropriate antibiotics to select against plasmid-free cells. After overnight incubation, the culture was diluted 10^{-6} fold into D/M minimal medium and grown into stationary phase. The cycle of dilution and growth was performed 5 times and each cycle represented 20 generations ($2^{20}=1x10^6$). From each stationary phase culture, samples were diluted and plated onto iso-sensitest agar and the resulting colonies tested for plasmid content via the B-Lactamase plate assay (see above). Around 1000 colonies were tested every 20 generations and after 100 generations the limit of detection should be 10^{-5} $\overset{\text{per cell per generation}}{\wedge}$ i.e. $1p^-$ cell in 10^5 should be detectable. Generally single colony gel analysis was performed after stability testing to ensure that the structural integrity of the plasmids remained

unaltered.

Copy Number Estimation via Single Cell Resistance to Cephaloridine: Plasmid containing cells were grown selectively in D/M minimal medium to mid-log phase (10^7 cells/ml) unless otherwise stated prior to diluting and plating equal volumes onto iso-sensitest plates containing increasing levels of cephaloridine. Duplicate and frequently greater than 2 plates per concentration were used. Cell survival graphs were obtained by counting the number of colonies surviving at each concentration, obtaining the mean value for each and expressing this as a percentage of the p^+ cells surviving on iso-sensitest plates without cephaloridine. The presence of plasmids(Ap^r) was determined using the B-Lactamase plate assay.

In vivo Labelling for Copy Number Determination: 200ul of a culture grown selectively overnight in L-broth was inoculated into 2ml of M9 minimal medium and incubated for 2 hours on a shaker prior to the addition of 10uCi/ml ^3H thymidine together with 20ul of 20mM deoxyadenosine. Deoxyadenosine was added because the cells were *thy*$^+$ and required a source of deoxyribose-1-phoshate. After re-incubation for a further 3 hours, 500ul of the culture was pelleted at 12000g in a microcentrifuge. The DNA was isolated using a modification of the method used by Projan *et al* (1983). The cells were resuspended in 100ul of lysing buffer (20mM Tris pH8, 10mM EDTA, 20% (w/v) glucose and 20mg/ml lysozyme) and incubated at 37°C for 30 minutes. Lysis was effected by adding 100ul of 2% SDS with vigorous vortexing. The lysate was then freeze-thawed 2x at -70 to 37°C and then treated with proteinase K at 10ug/ml for 30 minutes at 37°C. 50ul of the lysate was added to 50ul of FSB and vortexed well prior to loading 50ul onto a 0.8% agarose gal. The gel was electrophoresed in E-buffer at 4V/cm usually until the blue dye was about to run off the bottom. When large plasmids were used, the gels were allowed to run for a longer period of time in order to obtain maximum separation of the open-circular plasmid form from the chromosome. The gels were stained in EtBr and viewed on a 260nm UV transilluminator. The well origins (containing chromosome), chromosomal bands, plasmid supercoiled and open-circular forms were excised (Weisblum *et al* 1979) and added to screw capped bottles containing 2ml of dH_2O. Gel slices of equivalent sizes from a DNA

free region of each gel track were treated likewise to serve as blank controls for each individual experiment. The gel slices were melted by placing the vials in a boiling water-bath for 10-15 minutes. Thereafter, 1ml aliquots from each tube were added to scintillation vials containing 10ml of "Atomlight" scintillant, the vials shaken vigorously and placed at 4°C in the dark 1 hour before estimating the incorporated radioactivity in the scintillation counter.

The copy number per genome equivalent was calculated as follows:

$$\text{Copy Number} = \frac{\dfrac{\text{plasmid cpm}}{\text{chromosome cpm}} \times \text{Size } \underline{\text{E.coli}} \text{ chromosome(kb)}}{\text{Size of plasmid(kb).}}$$

The mass of the E.coli chromosome is 3.8×10^3kb (Cooper and Helmstetter 1968). Typically, a minimum of 3 single colonies containing each test plasmid were labelled and the mean copy number per genome equivalent was calculated.

Transduction of polA12$_{ts}$ fad::Tn10 from PO810 to DS903 using P1(km^r)$_{ts}$ phage: The donor strain PO810 was grown to late-log phase (approx 1×10^9/ml) in L-broth and 10mM $CaCl_2$. The cells were then pelleted and resuspended in P1 adsoption medium (1/10 vol). The phage were added to 200ul aliquots of the donor bacteria and allowed to adsorb at room temperature and the infected cells were then incubated in L-broth overnight at 30°C. The cells were plated onto L-agar Km, 10mM $CaCl_2$ plates and incubated at 30°C overnight. Km^r lysogens were picked and grown-up in L-broth plus kanamycin and 10mM $CaCl_2$ at 30°C. After several hours growth the culture was shaken at 42°C for 90 minutes in order to allow the temperature sensitive P1 phage to lyse the cells. After chloroforming the culture for 30 minutes, the lysate was obtained by spinning down the cell debris at 10K (12000g) for 10 minutes. Lysate titres were generally between 10^9-10^{10} pfu/ml.

The recipient strain DS903 was grown in L-broth and 10mM $CaCl_2$ to mid-log phase (2×10^8 cells/ml), spun down and resuspended in 2 ml of P1 adsorption buffer. P1(Km^r)$_{ts}$ phage were added to 200ul aliquots of DS903 and left for 25 minutes at room temperature to adsorb. The cells were then spun down and washed with 1% sodium citrate 2x, to chelate Ca^{2+} which prevents re-adsorption of any P1 phages. The cells were

incubated overnight in L-broth plus 2% sodium citrate to allow expression of tetracycline resistance (polA12$_{ts}$ fad::Tn10, Tn10 carries Tc^r). The "transductants" were then plated onto L-plates containing 10ug/ml tetracycline and incubated at 30°C. As a control, the P1 lysate was treated likewise, to check that no viable cells remained.

Tc^r colonies arising on the plates were firstly streaked onto minimal medium plus supplement plates in order to demonstate that they possessed a DS903 background and not the donor strain background (PO810). The Tc^r isolates were also Km^r indicating that they still possessed the P1(Km) lysogen, however repeated streaking at 42°C cured the cells of the phage.

To demonstrate that the DS903 Tc^r isolates were also polA12$_{ts}$, the cells were streaked onto L-plates containing 0.04% MMS (methylmethanesulphonate) at 42°C since polA12$_{ts}$ bacteria are sensitive to this mutagen (Monk and Kinross 1972). Two MMS^s isolates were obtained. To further determine if they were polA12$_{ts}$, the ColE1-derivative plasmids, pUC8 and pCJ112 were introduced by transformation at 30°C. The strains transformed with very low efficiency; a feature of polA12$_{ts}$ cells (D.Sherratt pers. comm.). Incubating the Ap^r transformants selectively at 42°C produced no growth, however, growth could occur under non-selective conditions at 42°C. Therefore the ColE1 derivatives failed to replicate at the non-permissive temperature but could do so at 30°C. This demonstrated that DS903 polA12$_{ts}$ strains had been constructed.

P1 Transduction of Spectinomycin Resistance to DS903: The donor strain DS835 Spc^r Stp^s was grown to mid-log phase in L-broth, the bacteria pelleted at 10K (12000g) and resuspended in a small volume of R-broth plus $CaCl_2$ and 100mM $MgSO_4$. Sufficient P1 phage to produce an m.o.i. of 0.001 were added to the bacteria and allowed to adsorb for 25 minutes at 37°C. 500ul of infected cells were added to 3ml of R-top agar and poured over very thin, wet L-agar plates. These were incubated in a sandwich box containing a small beaker of water for 6-8 hours only. After this time, substantial lysing of the bacteria had ocurred. The lysate was scraped off the plate by a glass spreader and transferred to a universal container. A few drops of chloroform were added and the lysate vortexed vigorously to kill and lyse remaining cells. The lysate was placed on ice for 30 minutes to solidify the agar particles prior to

pelleting them by spinning at 18k for 10 minutes at 4°C (39200 g). The supernatent was removed, titred and found to have 10^9 to 10^{10} pfu/ml.

The recipient strain DS903 was grown to mid-log phase ($2x10^8$ cells/ml) in L-broth plus 10mM $CaCl_2$ and the cells pelleted at 10K (12000g) for 10 minutes. Sufficient phage to give an m.o.i. of 0.01-0.1 were added to 200ul aliquots of the bacteria which were resuspended in P1 adsorption buffer and allowed to stand at room temperature for 25 minutes. The cells were then pelleted and washed 2x in 1% sodium citrate, resuspended in 10ml L-broth plus 2% sodium citrate and incubated overnight to allow expression of spectinomycin resistance. By plating on spectinomycin containing plates, several Spc^r isolates were obtained. These cells were patched onto streptomycin containinig plates to check that the closely-linked Spc^r Stp^r markers had been exchanged during transduction. All Spc^r isolates except one were Stp^s. The background of the isolates was checked by streaking onto minimal medium plus supplements and was found to be DS903 indicating that they were indeed genuine transductants.

Continuous Culture: The apparatus used was essentially that of Baker (1968). Media was fed into the 600ml working volume culture vessel from 20 litre media tanks via a Watson-Marlow peristaltic pump. The dilution rate (D) ($ml\,hr^{-1}$) was initially measured by the flow rate of media leaving a 10ml pipette attached to the apparatus via sterile silicon rubber tubing. When excess culture began to be removed via syphon overflow, the rate of discharge was measured periodically to ensure that D had not altered. The temperature was maintained at 37°C by a contact thermometer which controlled a heating lamp. Sterile air was delivered into the vessel via a fish tank pump connected to a flow meter. The culture was agitated by a magnetic stirrer. Sampling was carried out using a sterile hypodermic syringe via a Luer-Lok needle situated in a Suba-Seal rubber attachment on the chemostat. An automated pH control was not possible with the design of the apparatus, therefore a well buffered growth medium was used instead. Competition experiments between plasmid-free and plasmid-containing cells were performed as following unless otherwise stated: using two chemostats, the two test strains were initially grown into late-log phase prior to switching on the media pumps and allowing the culture to become "continous". The chosen dilution rate D was 0.4 and after several hours of continous growth,

300ml from each chemostat (600ml working volume) was inoculated into the other chemostat thereby producing mixed cultures. Samples were removed periodically, the cells plated onto isosensitest agar and the proportion of plasmid-free cells at each time point was calculated using the B-Lactamase plate assay.

2.10 In Vitro Techniques

Cleared Lysate DNA Preparation: 200 ml cultures of selectively grown plasmid containing cells were pelleted at 10K (12000g) at 4°C for 5 minutes. The pellet was resuspended in 3.3 ml of cold 25% sucrose 50mM Tris pH8 and put on ice for 5 minutes. The cell suspension was sphaeroplasted by mixing with 0.67 ml fresh lysozyme solution, 20 mg/ml in 250mM Tris pH 8.0 and incubated on ice for 10 minutes. Then 1.3 ml of 250mM Na_2EDTA pH 8.0 was gently mixed in and incubated on ice for a further 10 minutes. The protoplasts were lysed by the addition of 5.3 ml lytic mix solution mixed by inversion and incubated on ice for 30 minutes. The crude lysate was cleared by centrifugation at 18K at 4°C for 30 minutes (39200 g). This separated the cell debris as a spongy pellet from the supernatant which contained the plasmid DNA. The latter was carefully decanted and deproteinised by two phenol extractions (5 ml) followed by ether extraction of trace phenol. The plasmid DNA was purified by CsCl/EtBr gradient centrifugation: 5g CsCl was dissolved in 4.83 ml cleared lysate and 0.33 ml EtBr (3 mg/ml), producing a density of 1.58 g/ml. The mixture was poured into a 10 ml polypropolene ultracentrifugation tube, topped up with paraffin oil and centrifuged at 48K (200 000g) at 15°C for 16 hours. Two DNA bands were visible, a lower supercoiled plasmid and an upper chromosomal band. The plasmid specific band was removed with a hypodermic syringe, by puncturing the tube at the appropriate point. The EtBr was removed by repeated extraction with butanol and the resulting CsCl solution diluted 1 in 4 with TE buffer and the DNA precipitated by ethanol. The concentration of plasmid DNA was estimated from gels against a known concentration standard.

Birnboim-Doly DNA Purification: This method was modified by Chris Boyd in this laboratory. 200 ml cultures of plasmid containing cells were pelleted by centrifugation at 10 K for 5 minutes at 4°C in a Beckman

JA2-21 JA14 rotor (12000 g). The pellet was resuspended in 8 ml of lysis solution (Birnboim Doly I) and incubated for 5 minutes on ice. 16 ml of alkaline-SDS solution (Birnboim Doly II) were mixed in and the lysate left on ice for 5 minutes. 12 ml cold of 5M acetate solution (Birnboim Doly III) was added and mixed by inversion; incubation was continued on ice for 10 minutes. The viscosity decreases sharply as a white floccular precipitate is formed. This was removed by centrifugation in a Beckman JA2-21 JA20 rotor at 18 K for 30 minutes at 20°C (39200 g). The 18 ml (approximate) cleared supernatant was then deproteinised by phenol extraction (6 ml) followed by ether extraction of trace phenol. The plasmid DNA was preciptated at room temperature to avoid excessive coprecipitation of SDS with 12 ml isopropanol for 15 minutes. The DNA was pelleted in a Beckman JA2-21 JA20 rotor for 30 minutes at 18K, 20°C (39200 g). After decanting the supernatant the nucleic acid pellet was rinsed vigorously in cold 70% ethanol. The DNA was further purified by banding on a CsCl/EtBr gradient. The pellet was resuspended in 2.09 ml TE to which 4.324 ml TE containing 5g CsCl and 270 ul 15 mg/ml EtBr was added and mixed. The solution was transferred to a polypropolene 10 ml ultracentrifuge tube, the extra volume being made up with paraffin oil. The gradients were centrifuged in a Beckman Ti70 fixed angle rotor at 49K (200 000 g) 15°C for 16 hours. The plasmid DNA was purified as above. After butanol extraction, the DNA was dialysed against 1xTE to remove the CsCl.

Mini-DNA Preparation: Restrictable and clonable DNA was isolated using a modification of the method of Holmes and Quigley (1981). An overnight 10ml culture was pelleted at 10K (12000 g) for 5 minutes at 4°C. The pellet was resuspended in 700ul of ZIPPY solution and transferred to a 1.5 ml Eppendorf tube. 50 ul of a 20 mg/ml solution of lysozyme in 250mM Tris pH8 was added and the tube placed in a boiling water bath for 45 seconds to lyse the cells. The cell debris and chromosomal DNA was separated from the plasmid DNA by 15 minutes centrifugation in a microcentrifuge (12000 g). The supernatant containing the plasmid was phenol extracted 2x to deproteinate it and then chloroform extracted 2x, spinning at 12000g between each stage. The supernatent was then decanted into an equal volume of isopropanol and placed on ice for 15 minutes to precipitate nucleic acid. The DNA/RNA was pelleted by 15 minutes centrifugation in a microcentrifuge,

dried under vacuum and resuspended in 50 ul TE buffer. RNAase treatment was required prior to loading DNA restrictions onto gels.

Ethanol Precipitation of DNA: In many experimental procedures DNA must be treated serially with different enzymes requiring different buffers. The most convenient method of changing the buffer is to ethanol preciptate the DNA from solution and resuspend the dried DNA pellet in the second. If required, proteins were removed by phenol extraction and then traces of phenol removed by ether extraction before ethanol precipitation. The solution was made 0.3M in sodium acetate by addition of 1/9th volume 3M sodium acetate pH 5.3. To this was added 2.5 volumes of ethanol. Precipitations were carried out at -20°C for 30 minutes to 24 hours unless otherwise indicated. For rapid precipitation, a dry ice methanol bath -70°C was used for 15 minutes. The DNA was pelleted by centrifugation at 12000 g in a microfuge for 15 minutes or Beckman JA2-21 JA20 rotor for 30 minutes at 18k at 0°C (39200g). After decanting the supernatant, traces of precipitated salt were removed by a cold (-20°C) 70% ethanol wash. Finally, the pellet was dried down under vacuum.

Rapid Purification of DNA on Sephadex G-50 Columns: This simple technique was used to ensure that mini prep DNA was restrictable, it was developed by David Summers in this laboratory. A 0.4 ml Eppendorf tube was punctured at the base and filled with 20 ul of siliconised acid washed sand followed by 300 ul of G-50 equilibrated in TE. The column was packed by centrifugation inside a 1.2 ml eppendorf tube. It was washed twice in 150 ul TE buffer driven through by centrifugation. The DNA solution was applied to the nearly dry column and centrifuged through for approximately 1 minute. Because the column is dry, no ethanol precipitation is required and the DNA is ready for use.

Single Colony Gels: This technique enables the plasmid content of an isolate to be observed without the need to isolate DNA. A single colony was patched out (1cm square) on an selective plate and grown overnight. Using a toothpick a large scrape of cells was collected and resuspended in 100 ul of Single Colony Gel buffer. The cells were left to lyse at room temperature for 15 minutes. Cell debris and chromosomal DNA were separated by centrifugation in a microcentrifuge for 15 minutes (12000

g). 50 ul of the supernatant containing the plasmid DNA was loaded on to an agarose gel.

Restriction of DNA: Restriction of DNA was routinely carried out in 0.4 ml Eppendorf tubes. Reactions were usually performed in a total volume of 20 ul containing 0.5 to 2.0 ug of DNA, 2 ul 10x restriction buffer and 0.5 to 2.0 units of enzyme, the volume being made up with gelatin-TE buffer. The reactions were incubated at the appropriate temperature as recommended by the suppliers for 1 to 3 hours after which time digestion was usually complete. Restriction was arrested by either the addition of gel loading buffer or if serial treatments were required, phenol extraction followed by ethanol precipitation. This avoided the problem of the first enzyme inhibiting the second. Digestions involving Lambda DNA for size markers were usually heated at 65°C for 5 minutes to dissociate the cohesive ends prior to loading on a gel.

Ligation: T4 DNA ligase catalyses the formation of covelently joined hybrid DNA molecules from "sticky" (complementary) ended single stranded DNA molecules. Under conditions of high DNA concentration it can similarly catalyse the ligation of "blunt" ended (no single stranded extension) precursors. Ligations of both sorts were carried out in this work. The plasmid cloning vector was first digested with the desired restriction enzyme and mixed with compatible restriction fragments of the target DNA. The concentration of fragment to vector was adjusted to approximately 3 to 1 for sticky and 10 to 1 molar excess for blunt ended clonings. Blunt ended clonings required a higher molar excess of fragment and a longer incubation period approximately 16 hours at 16°C. Sticky ended clonings could be effected in 4 hours at 16°C. A typical 20 ul reaction mix comprised about 50 ng of DNA (vector and fragment), 2ul 4mM ATP, 2ul 10x ligation mix and 0.01 for sticky or 0.1 units for blunt ends of T4 DNA Ligase. The volume was made up to 20ul in TE buffer. For blunt ended clonings the reaction volume was reduced to 10 ul, all components except the amount of DNA were adjusted accordingly.

Phosphatase Treatment: (1) CIP. Self ligation of cloning vector molecules reduces the efficiency of cloning dramatically. By removing the 5' terminal phosphate groups on linearised vector prior to mixing

with target fragment, self ligation is minimised since T4 DNA ligase requires a 5' terminal phosphate on one precursor molecule. Calf intestinal phosphatase (CIP) operates in high, medium and low salt restriction buffers; about 0.01 units were added to a vector DNA restriction digest and incubated at 37°C for 15 minutes. It is heat labile (68°C) but was usually removed along with the restriction enzyme by phenol extraction.

(II) **BAP.** The restricted vector DNA was resuspended in 50ul 10mM Tris pH8 and 20 units of BAP added for each ug of DNA. The mixture was incubated at 65°C for 1 hour and the enzyme subsequently removed by extracting 3x with phenol, followed by extracting 3x with diethyl-ether. The DNA was then ethanol precipitated and used for ligation.

Nick Translation: E.coli DNA polymerase I has a 5' to 3' polymerase and a 3' to 5' and 5' to 3' exonucleolytic ability. Therefore, in addition to the polymerisation of nucleotides onto a 3' terminal hydroxyl group, it can also remove nucleotides 5' of a nick and resynthesise the chain. By replacing a proportion of the free nucleoside bases with [a-^{32}P]dATP, it is possible to prepare radiolabelled DNA fragments with a high specific activity. A typical nick translation comprised 0.5 to 1.0 ug of restricted DNA, 2 ul of 1mM solution of each dXTP, 20 uCi [a-^{32}P]dATP 1000 Ci/mMole, 5 ul 10x Nick Translation buffer, the volume was made up to 50 ul with sterile distilled water and chilled on ice. Nicks were introduced by the addition of 0.5 ul of DNAase I (0.1 ug/ml) incubated at 16°C for 15 minutes prior to the addition of 5 units DNA polymerase I. This was incubated for one hour at 16°C. Polymerisation was arrested by the addition of 2 ul 500mM EDTA. The unincorporated nucleosides were separated by a Sephadex G-50 5ml column. To check that separation was effective, duplicate 1ul aliquots of the column fractions were applied to Whatman GF/C glass-fibre discs and allowed to dry. One of each duplicate disc was submerged in 5ml 10% trichloroacetic acid (TCA) on ice for 30 minutes and then washed in methanol and allowed to air dry. This process was repeated 3x. The efficiency of separation and percentage of incorporation was deduced by Cherenkov counting the washed and unwashed discs.

Southern Transfer and Hybridization.: Southern transfer was performed by a modification of the proceedure described by Southern (1979).

After EtBr staining and photography, the gel was transferred to a dish and the DNA denatured by placing in 150ml of Gel Soak I for 30 minutes with gentle agitation. The buffer was then replaced by 150ml Gel Soak II in order to neutralise the gel and further agitated for 30 minutes. The gel was then placed onto 3mm filter paper on a glass support placed between two reservoirs containing 20x SSC. A piece of Biodyne A membrane was then placed on top of the gel and bubbles expelled by rolling a pipette over the membrane. One sheet of 3mm filter paper was placed over the membrane, followed by a 3 inch stack of paper towels, a glass plate and a 1kg weight. Transfer was allowed to proceed overnight. The membrane was then baked in an 80°C oven for 1 hour. At this stage, the agarose gel was restained with EtBr to check that transfer had been efficient. After baking, the membrane was pre-hybridized with non-homologous DNA (salmon sperm) which had been denatured by the addition of 1/10 vol. 1M NaOH and heating at 65°C for 10 minutes. After denaturation, the DNA was neutralized by adding 1/10 vol. 1M HCl. The membrane was sealed in a plastic bag with 4ml/100cm^2 of pre-hybridization fluid and immersed in a 65°C water bath for 1 hour. After pre-hybridization at 65°C, the plastic bag was opened and the solution removed and replaced by hybridization fluid (2ml/100cm^2) which is the same as pre-hybridization mix except that ^{32}p-labelled probe is added to it. The bag was resealed and immersed at 65°C once more overnight. After hybridization, the membrane was dipped into Wash Buffer, placed in a fresh plastic bag with 250ml Wash Buffer/100cm^2 of membrane and agitated for 30 minutes at room temperature. The Wash Buffer was then discarded. The washing proceedure was repeated 2x more, the filter was dried at 37°C, placed on a glass plate and a sheet of X-omat film laid on top. A tungsten intensifying screen was then placed on top of the film and the light tight cassette sealed.

2.11 DNA Electrophoresis Through Gels

Two gel matrices were used in this work, for DNA of 1 kb and above, agarose gels were employed, 1% for horizontal restiction gels and 0.8% for single colony gels. For DNA fragments of less than 1 Kb, polyacrylamide gels were used at a concentration of 5.0%.

Agarose Gels: HORIZONTAL RESTRICTION GELS. Agarose powder (gelling

temperature 36-42°C) was dissolved at 100°C in 100 ml of 1x E buffer in a perspex flask. The molten agarose was cooled to 55°C prior to the addition of 20 ul EtBr 3mg/ml. The perspex gel former mounted on a flat pouring bed was sealed with autoclave tape with a teflon well former located at one end. The gel was poured and allowed to solidify. Once the wells were wetted with E buffer, the comb was carefully removed. The gel was removed from the gel former and transferred to the gel running apparatus. This comprised two 500 ml buffer tanks separated by a raised platform upon which the gel was placed. Sufficient 1x E buffer was supplied to just cover the gel. The samples were loaded using a 5-50 ul Finnpipette. Gels were usually run at 5 Volts/cm giving a run time of around 3 hours.

VERTICAL GELS: Gel kits which held 16x15 cm glass plates separated by 3mm spacers were used for single colony gels. 100ml of molten agarose (0.8%) pre-cooled to 55°C was poured between the glass plates after these had been sealed with 10ml of agarose. 10 or 15 toothed teflon combs were removed carefully when the gel had set, after the addition of 1x E buffer to both top and bottom resrvoirs. A typical single colony gel is run at a maximum of 6.6V/cm for around 3-4 hours. Gels were stained in EtBr (0.5ug/ml) for 30 minutes prior to being photographed on a 260nm UV transilluminator using a Polaroid camera loaded with Polaroid 4x5 Land Film (no. 57) or a Pentax 35mm SLR loaded with Ilford HP5 film; both were fitted with a Kodak Wratten Filter No. 9 (red).

The interpretation of gel tracks of untreated plasmid DNA were based on Dugaiczyk *et al* (1975). The fastest migrating band was the supercoiled plasmid monomeric DNA which was the most abundant band. Behind this ran an open circular plasmid (DNA) and often comigrating supercoiled plasmid dimeric DNA (2^{m}SC). Open circular dimers and higher forms if visible ran even more slowly. Plasmid linears ran between the SC and OC plasmid forms. In single colony gels, sheared fragments of chromosomal DNA ran as a single thick band towards the top of the gel. The multimeric state of plasmids isolated from *rec*$^{+}$ cells was determined by using super-coiled size markers.

Polyacrylamide Gels: The gel was polymerised in a vertical gel kit between two glass plates 15x16 cm separated by 1.5 mm spacers, using a teflon comb to form the wells. The gel apparatus was sealed with 1x TBE

1% agarose. The stock solution of acrylamide was 20% Acrylamide/1% bis (w/v).

	5.0%
Acrylamide Stock:	5.0 ml
10x TBE pH 8.3	6.0
distilled water	37.9
10%$(NH_4)_2S_2O_8$	0.72
TEMED 10%	0.36

The gels were run at constant current (25 mA) in 1x TBE at room temperature. DNA bands were visualised by staining in 0.5 ug/ml EtBr for 30 minutes and photographed as above.

Sizing of Restriction Fragments: The distance migrated by a DNA fragment is related to its size such that small molecules migrate the furthest. Hand measured mobilities of restricted linear DNA were entered into a computer program known as ZGELFIT. This calculates the molecular size relative to a previously entered set of standards. This method has been shown to be empirically superior to methods plotting mobility against log molecular weight (C. Boyd PhD thesis Leicester 1983). Additionally, it provides the standard deviation of the variance between the actual and calculated molecular sizes. In this way, one can assess the accuracy of measurements prior to entering the test data and remeasure if necessary.

2.12 Extraction of DNA From Gels

Agarose Gels:(1). Tautz and Renz (1983). An ethidium stained gel was visualised on a long wave transilluminator (300 to 360 nm) and the band(s) required excised. The gel chips were transferred to a 0.4 ml Eppendorf tube punctured at the end with a siliconised glass wool plug and sealed on the outside with parafilm. It was equilibrated in 10x its' volume of Tautz Renz I for approximately 40 minutes at 37°C in the dark. The tube was transferred to a -70°C dry ice/ methanol bath for 5 minutes. The 0.4 ml Eppendorf tube was unsealed and placed inside a 1.4 ml Eppendorf tube and immediately centrifuged in a microcentrifuge

for 15 minutes (12000 g). The DNA/ Acetate complexes were spun through the frozen gel matrix and trapped in the 1.5 ml Eppendorf tube. The remains of the gel was trapped in the glass wool filter. To the eluted DNA solution 1/100 volume of Tautz Renz II and 2.5x volume Ethanol were added. The DNA was precipitated at -70°C for 15 minutes and pelleted by a 15 minute spin in a microcentrifuge. The DNA/ salt pellet was washed in cold Tautz Renz III and repelleted by 5 minutes centrifugation. The pellet was finally washed in 100% Ethanol and vacuum dried. It was resuspended in 15 ul TE and subjected to 2 minutes centrifugation to remove traces of contaminating agarose.

(2), Electro-elution. After staining, the gel was placed on a long-wave transilluminator (300-360nm) and the band of interest excised. The gel slice was sealed in a short length of dialysis tubing along with 100ul of TE buffer. The dialysis tubing was placed in a horizontal gel kit and secured using plasticine prior to the addition of 1x E-buffer. The slice was electrophoresed at 50V for 3 hours. The current was then reversed for 30 seconds to remove DNA from the sides of the tubing. The dialysis bag was opened, the TE removed and the DNA precipitated with ethanol. The DNA was then ready to be used for transformation or cloning.

(3).**Low Melting Point Agarose:** The band of interest was excised from a 1% low melting point gel, melted at 65°C and diluted and mixed with 2 volumes of E-buffer also at 65°C. The mixture was then cooled to 37°C and an equal volume of distilled phenol (room temp) added and vortexed vigorously. The mixture was spun at 12000g in a microcentrifuge for 2 minutes to separate the aqueous and phenol phases. A white layer is formed at the interface and contains the agarose. Care must be taken not to remove this along with the supernatant since ions in the agarose interfere with ligations etc. The supernatant was chloroform extracted 2x and the DNA precipitated with ethanol.

(4).**Acrylamide Gels:** The DNA was electro-eluted as above using 1x TBE buffer instead of 1x E-buffer.

CHAPTER 3

ANALYSIS OF THE STABILITY AND COPY NUMBER OF pAT153 AND pBR322 MULTIMERS.

INTRODUCTION

For plasmids to be maintained within a population of cells their replication must be controlled so that the replication rate can be adjusted to the growth rate of the cells or cell division must be stalled until sufficient replication has occurred. Subsequent partitioning of plasmid copies at cell division by either an active or passive mechanism, need only ensure that both daughter cells receive at least one plasmid copy. The replication control requirement is fulfilled for many plasmids by a negative feedback loop, involving plasmid encoded *trans-acting* inhibitor substances such as small anti-sense RNA species (Scott 1984).

Although many low copy number plasmids are actively partitioned, no active partitioning mechanism has been demonstrated for the multicopy plasmid ColE1. For this plasmid, stable maintainance is thought to result from the high copy number which is maintained by *cer* the *cis*-acting site at which site-specific recombination acts to convert multimeric molecules to monomers. Random partitioning at cell division then ensures that each daughter cell receives a copy of the plasmid (Summers and Sherratt 1984; Summers and Sherratt 1985).

The vectors pAT153 and pBR322 have reputedly high copy numbers and have been reported to show some instability. This chapter investigates the stability and copy number of monomeric and multimeric forms of pAT153 and pBR322 and discusses factors which could lead to instability of high copy number cloning vectors.

RESULTS AND DISCUSSION

3.1 Isolation of Multimeric Forms of pAT153 and pBR322.

In an attempt to isolate multimeric forms of pAT153 and pBR322 (Fig 3.1A and B), monomeric plasmid DNA was transformed into JC8679 (*recBC sbcA*), a strain hyper-recombinogenic for plasmids (Fishel *et al* 1981).

Figure 3.1A

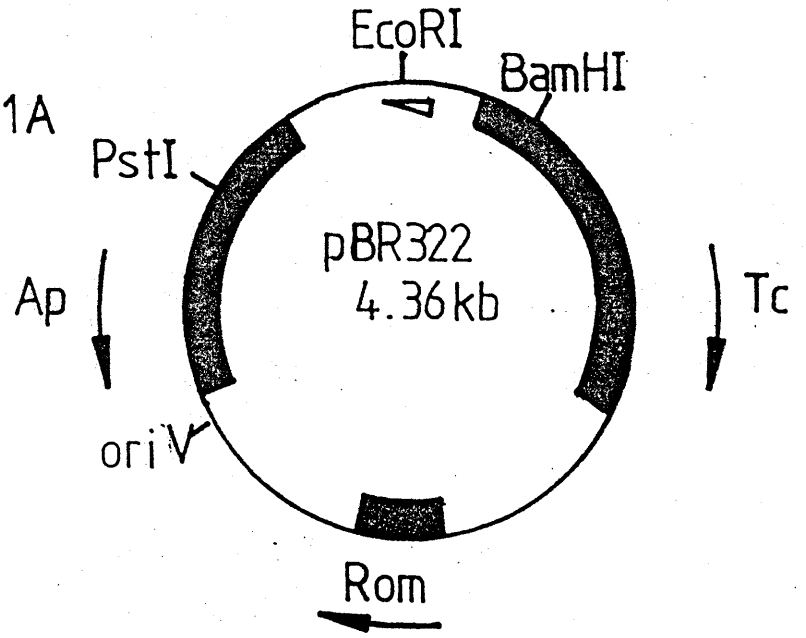
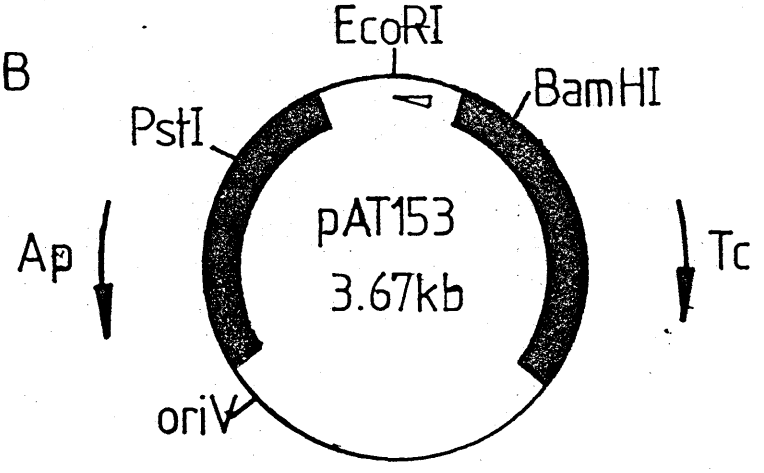


Figure 3.1B



direction of transcription

"anti-tet" promoter

Figure 3.1A + B Plasmid pBR322 and pAT153. Schematic diagram of pBR322, showing the location and direction of transcription of the Ap^r, Tc^r and *rom* genes. The position of the "anti-tet" promoter is also shown. Figure 3.1B is a schematic diagram of the pBR322 deletion derivative pAT153 which is deleted for *bom*, the region required *in cis* for transfer and *rom*, a copy number control gene.

Figure 3.2A

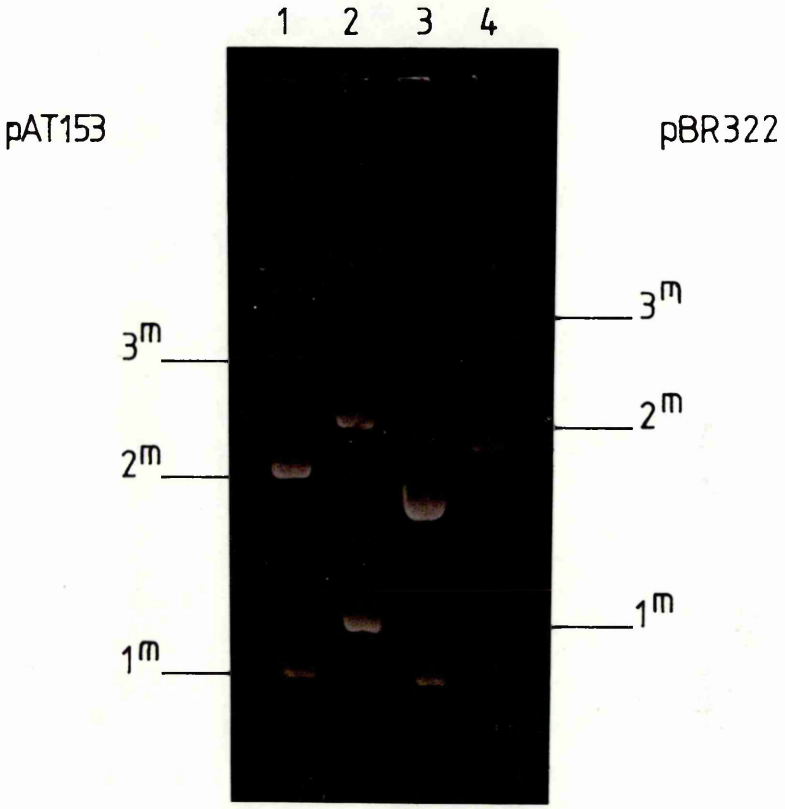


Figure 3.2A Plasmid Multimers. 0.8% agarose gel showing multimeric forms of pAT153 and pBR322 in the hyper-recombinogenic host JC8679.

(1) JC8679 pAT153
(2) JC8679 pBR322
(3) pAT153 1^m marker
(4) pBR322 1^m marker

Figure 3.2B

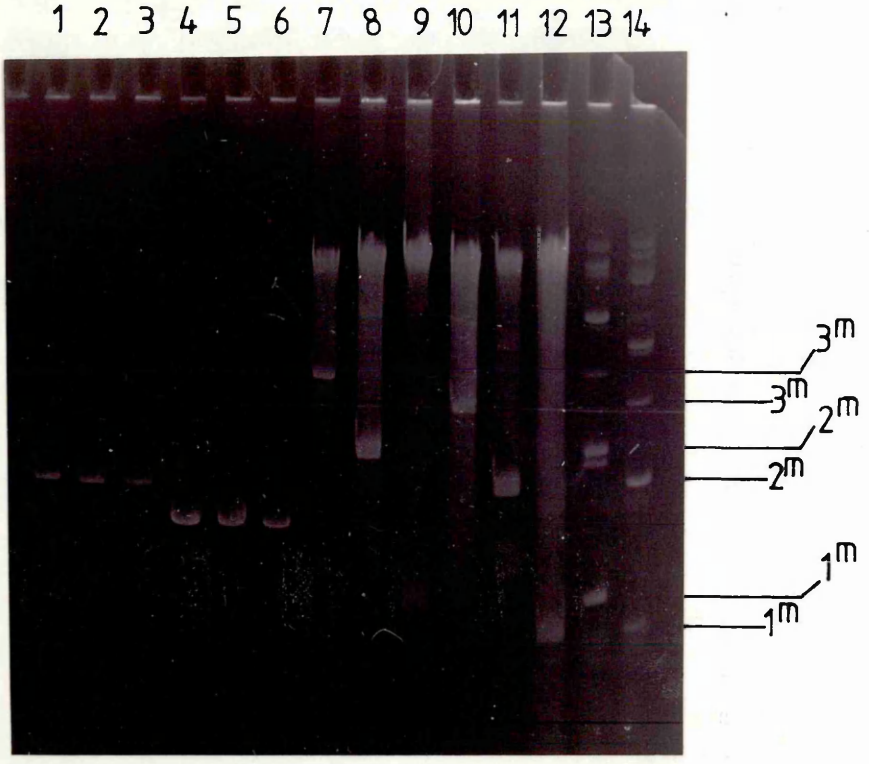


Figure 3.2B Specific Multimeric Forms maintained in DS903. 0.8% agarose gel showing pAT153 and pBR322 linearized by EcoRI to the unit monomeric size. Specific multimeric forms of pAT153 and pBR322 are also shown.

(1) pBR322 3^m EcoRI
(2) pBR322 2^m EcoRI
(3) pBR322 1^m EcoRI
(4) pAT153 3^m EcoRI
(5) pAT153 2^m EcoRI
(6) pAT153 1^m EcoRI
(7) DS903 pBR322 3^m
(8) DS903 pBR322 2^m
(9) DS903 pBR322 1^m
(10) DS903 pAT153 3^m
(11) DS903 pAT153 2^m
(12) DS903 pAT153 1^m
(13) JC8679 pBR322
(14) JC8679 pAT153

Specific multimeric forms were isolated from agarose gels (Fig.3.2A) and used to transform DS903 (a recF derivative of AB1157) to ampicillin and tetracycline resistance. The transformants were screened by single colony gel analysis to determine the multimeric state of the plasmid (Fig.3.2B). Monomers, dimers and trimers of pAT153 and pBR322 were isolated and their sizes checked using super-coiled DNA standards (Fig. 3.3). Mini-preps of plasmid monomer and multimer DNA were restricted with EcoR1, a unique cutting enzyme to demonstrate that the multimers were composed solely of monomeric units. The strain DS903 was chosen because its recF genotype renders it a suitable host for the "trapping" of plasmid multimers since plasmidic recombination is minimal in recF strains (Cohen et al 1984). A recA strain would also have been suitable, however, its growth rate in minimal glucose, casamino acid medium during stability experiments was much slower than the recF strain.

3.2 Do Multimers of pAT153 and pBR322 Have Lower Copy Numbers Than the Monomeric Species?

If the Pritchard "origin counting" hypothesis is correct, then one could imagine that plasmid multimers would be maintained at a lower copy number than plasmid monomers. Dimer molecules containing two replication origins would be maintained at half the copy number of the monomeric species and similarly trimers at one third of the monomeric level. Initially, the copy numbers of the multimeric forms of pAT153 and pBR322 were estimated using single cell resistance to antibiotics. Uhlin and Nordstrom (1977) demonstrated a linear relationship between resistance to ampicillin and B-lactamase gene dosage, using R plasmid copy mutants. Since pAT153 and pBR322 are both resistant to ampicillin because they encode the Tn3-derived bla gene, it was possible to use this technique to estimate their relative copy numbers. The degree of resistance of Ap^r plasmid containing strains is obtained by plating the cells onto increasing concentrations of ampicillin and obtaining cell survival curves. The LD_{50} values of each strain can then be compared.

Since a plasmid with a copy number of 10 per genome equivalent survived on almost 2mg/ml ampicillin (Uhlin and Nordstrom 1977) and published estimates of pBR322 quote copy numbers of between 40-50 per genome equivalent (Timmis 1981), very large quantities of ampicillin would have been required for copy number estimations of these plasmids.

Figure 3.3

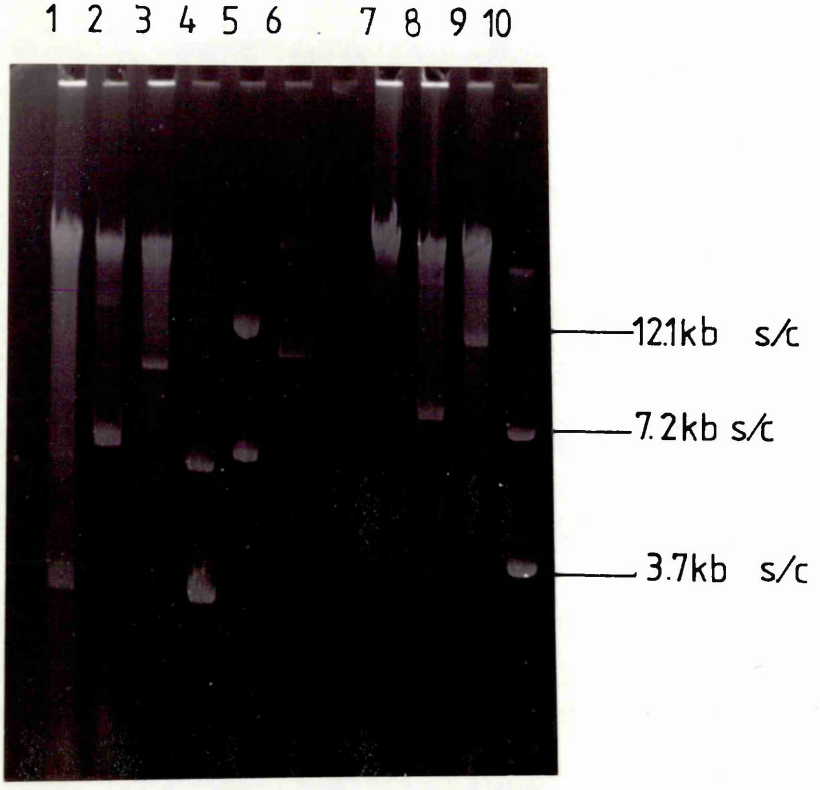


Figure 3.3 Sizing of Plasmid Multimers. 0.8% agarose gel showing supercoiled multimeric forms of pAT153 and pBR322 run alongside supercoiled size marker DNA.

(1) DS903 pAT153 1^m
(2) DS903 pAT153 2^m
(3) DS903 pAT153 3^m
(4) pAT153 1^m DNA
(5) ColK DNA 7.2kb
(6) pDS4101 DNA 12.1 kb
(7) DS903 pBR322 1^m
(8) DS903 pBR322 2^m
(9) DS903 pBR322 3^m
(10) pBR322 1^m DNA

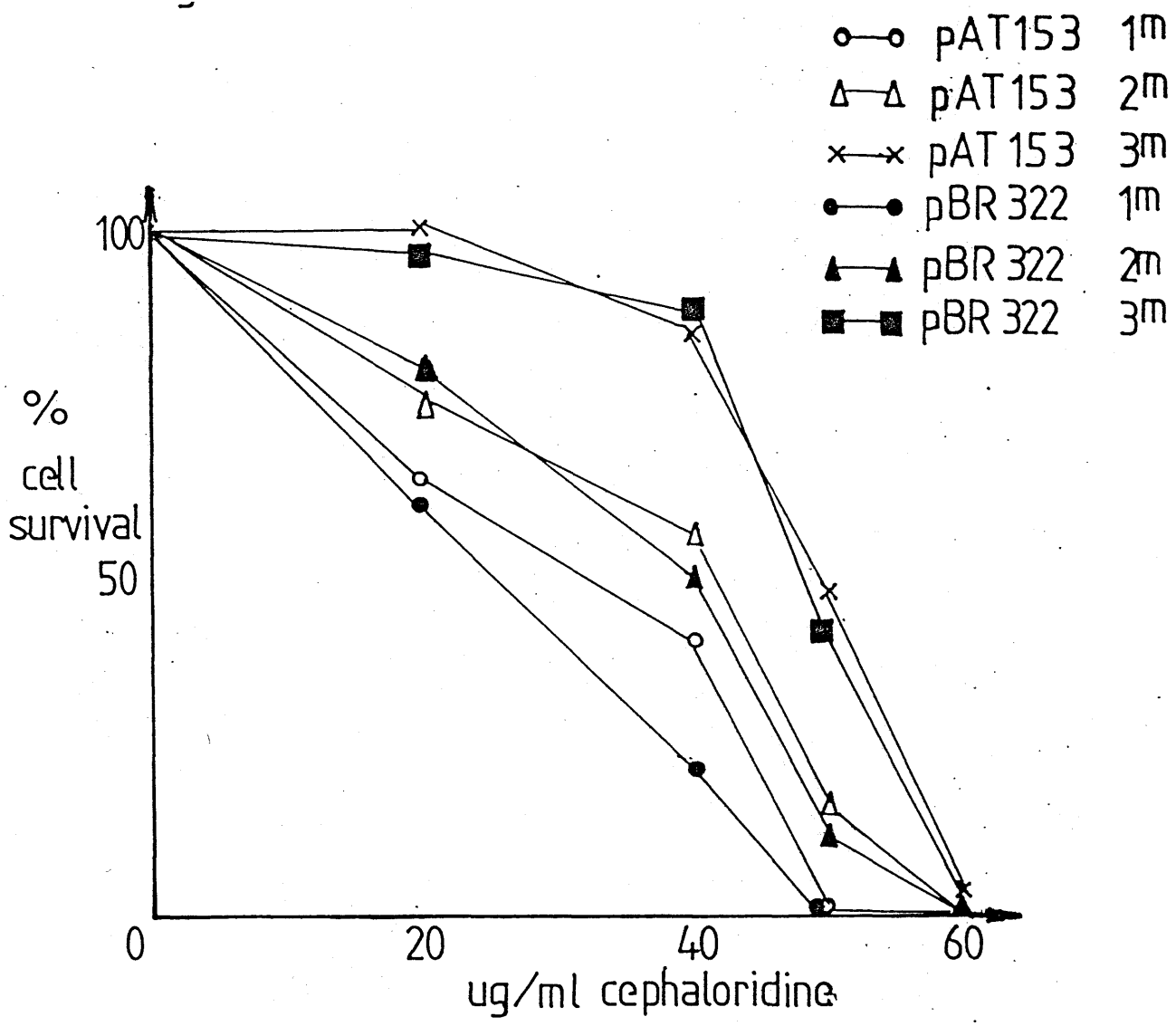


Figure 3.4 Single Cell Resistance to Cephaloridine. Killing curves of various exponentially growing plasmid containing strains. The LD_{50} values are shown below.

DS903 pAT153 1^m 31 ug/ml cephaloridine.
DS903 pAT153 2^m 41 " " "
DS903 pAT153 3^m 49 " " "
DS903 pBR322 1^m 25 " " "
DS903 pBR322 2^m 40 " " "
DS903 pBR322 3^m 48 " " "

Therefore, cephaloridine, a synthetic cephalosporin was used. Because of alterations in the cephaloridine B-lactam ring, it rapidly enters the periplasmic space where inactivation of the slower penetrating ampicillin occurs. Cephaloridineis more potent than ampicillin because the B-lactamase enzyme is unable to cleave all of the B-lactam rings and cell lysis occurs (Bryan 1980).

For each plasmid containing strain, exponential or stationary phase cultures grown in D.M. minimal medium were plated onto agar plates containing increasing concentrations of cephaloridine. The number of colonies growing on duplicate cephaloridine plates were expressed as a percentage of the Ap^r colonies growing on drug free plates.

The 2^m and 3^m plasmids contain 2 and 3 B-lactamase genes respectively. Consequently, if these multimers had the same copy number as the monomeric species and if a linear relationship exists between resistance and gene dosage for cephaloridine, this should be reflected in the single cell resistance data. The 2^m containing cells should be twice as resistant as the 1^m and 3^m containing cells should be three times as resistant. Alternatively, if the "origin-counting" hypothesis is correct and the 2^m has half the copy number of the 1^m and the 3^m one third of the 1^m value, then the resistance curves should be identical.

Figure 3.4 shows the resistance curves of the exponential phase cells containing pAT153 and pBR322 multimers. The graphs for the multimeric forms of pAT153 and pBR322 show LD_{50} values for pAT153 1^m, 2^m and 3^m of 31, 41 and 49ug/ml and 25, 40 and 48ug/ml for pBR322 1^m, 2^m and 3^m.

Figure 3.5 shows the resistance curves for stationary phase cells. The same general resistance trends observed for exponential phase cells are also observed for stationary phase cells. However, one difference is apparent, stationary phase cells seem to be more resistant to cephaloridine than their exponential phase counterparts. This could be due to two reasons. Firstly, stationary phase cells have a different cellular composition to exponentially growing cells. A long lag phase will occur when the cells are plated onto cephaloridine plates, during which time B-lactamase will continue to be synthesised. This may inactivate much of the cephaloridine in the immediate vicinity, resulting in reduced killing ability when the cell leaves log phase, begins to grow and becomes succeptible to attack. Secondly, Stueber and Bujard (1982) demonstrated that there was an inverse correlation between copy number and cell growth rate, such that the copy number was the

Figure 3.5

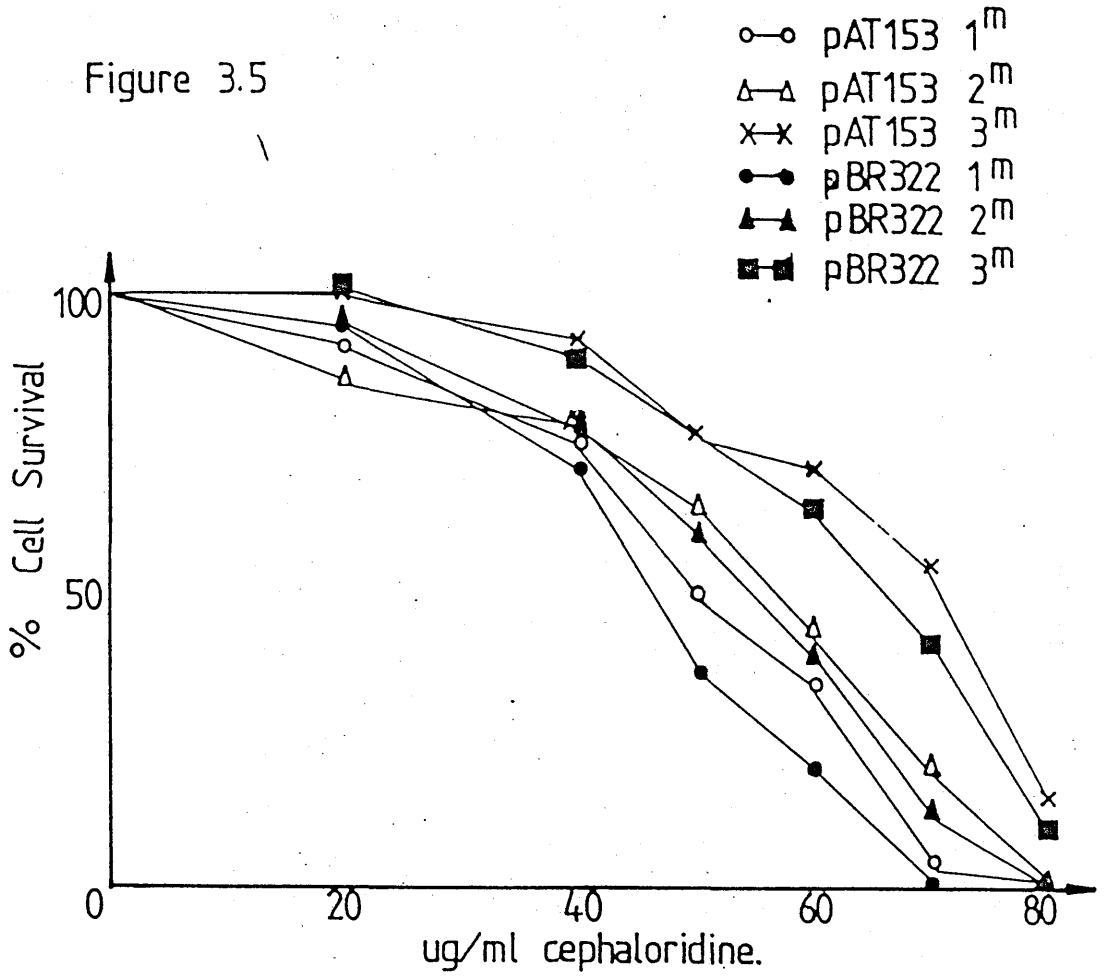


Figure 3.5 Single Cell Resistance to Cephaloridine. Killing curves of stationary phase plasmid containing cells. LD_{50} values are as follows.

DS903 pAT153 1^m	50	ug/ml cephaloridine
DS903 pAT153 2^m	56	" " "
DS903 pAT153 3^m	71	" " "
DS903 pBR322 1^m	45	" " "
DS903 pBR322 2^m	54	" " "
DS903 pBR322 3^m	65	" " "

highest in stationary phase cells. This may be explained by the rate of ColE1 replication being increased by cAMP, a cyclic compound whose intracellular concentration is depressed by the presence of glucose. Presumably in stationary phase, the glucose carbon source becomes limiting with the result that cAMP levels increase. A cAMP dependent promoter has been located in pBR322 at position 2270 (Sutcliffe 1978) and is conserved in ColE1 and ColK DNA sequences (J.Archer pers.comm.). Its position provides circumstantial evidence that it is involved in transcription of the mobility region (Katz et al 1973).

It is interesting to speculate upon the possibility that transcription from this promoter could interefere with transcription of the "counter-transcribed" rom gene. This would explain the increase in plasmid replication observed in the presence of high levels of cAMP and may account for the higher copy number of ColE1-like plasmids in stationary phase.

Assuming a linear relationship between resistance to cephaloridine and gene dosage, the LD_{50} comparisons indicate that the 2^m and 3^m forms of each plasmid have lower copy numbers than the 1^m form but they appear to be slightly higher than predicted by the "origin-counting" model (see concluding remarks). Finally, the copy number of pBR322 does not differ greatly from the copy number of pAT153, although published estimates show a 1.5-3 fold difference between pBR322 (rom$^+$) and the rom$^-$ derivative pAT153 (Twigg and Sherratt 1980).

3.3 Construction of Multimers Possessing Only One Functional Ampicillin Resistance Gene.

In order to determine more conclusively if multimers are maintained at lower copy numbers than monomers using single cell resistance to cephaloridine, multimeric molecules containing only one functional Ap^r gene were constructed. This involved constructing Ap^sTc^r and Ap^rTc^s pAT153 monomer molecules followed by restricting and ligating them together to form multimers.

3.3.1 CONSTRUCTION OF Ap^sTc^r pAT153 DNA.

pAT153 DNA was cut once in the bla gene with Pst1. ColE1 DNA was also digested with Pst1 which produced three restriction fragments of 5377, 1188 and 81 base pairs. The DNAs were ligated and used to transform

Figure 3.6A

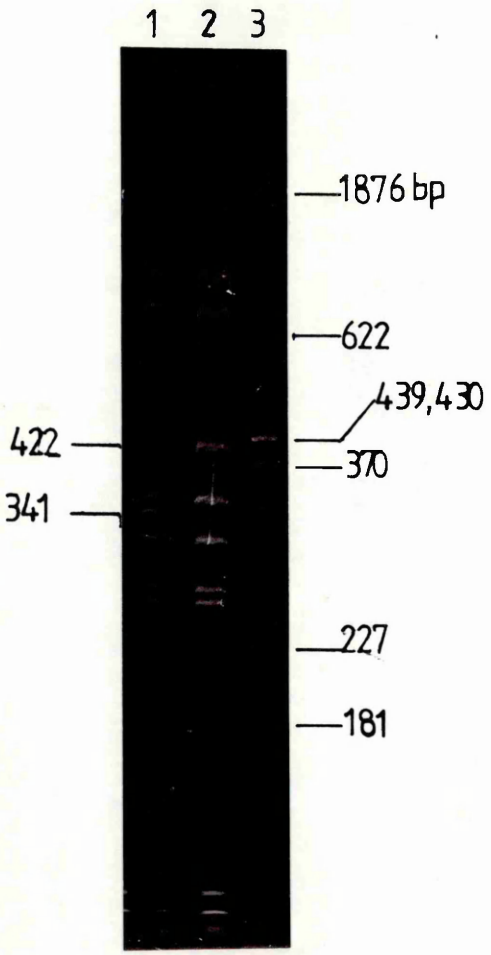


Figure 3.6A Sau 3A Restriction Analysis of pCJ104. 5% acrylamide gel showing the Sau3A profile of pCJ104 and pAT153. The 341bp Sau3A fragment which spans the unique PstI site in wild-type pAT153 has been replaced by a 422bp fragment in pCJ104, indicating that the 81bp ColE1 PstI fragment has been cloned into the PstI site, thereby inactivating the bla gene.

(1) wild-type pAT153 DNA Sau3A
(2) pCJ104 DNA Sau3A
(3) pBR322 size markers HaeII

Figure 3.6B

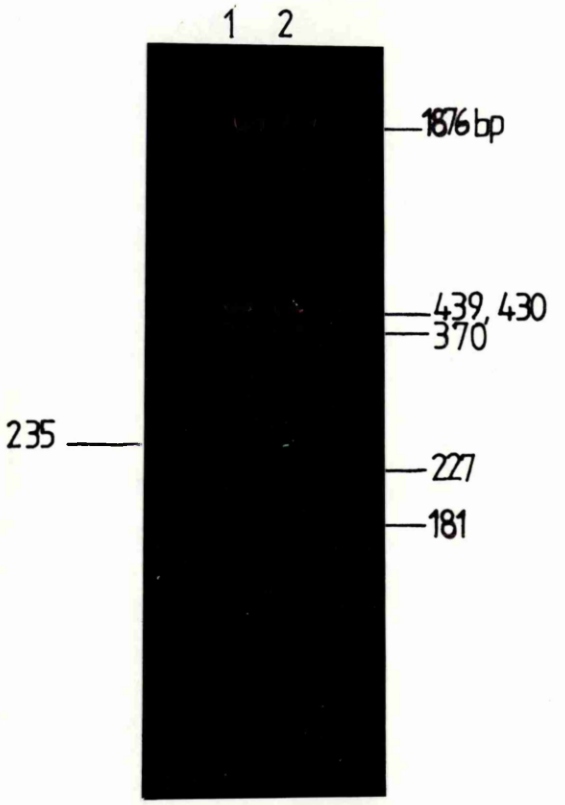


Figure 3.6B HaeII Restriction Analysis of pCJ103. 5% acrylamide gel showing the HaeII restriction profile of pCJ103 and pAT153. The 181bp HaeII fragment spanning the BamHI site has been replaced by a 235bp fragment in pCJ103 indicating that a 56bp ColE1 Sau3A fragment has been cloned into the unique BamHI site, inactivating the Tc^r region.

(1) pCJ103 HaeII

(2) pAT153 HaeII

DS903 to tetracycline resistance. The resulting Tc^r colonies were screened for ampicillin sensitivity and analysed on single colony gels. Several Ap^sTc^r colonies appeared to be a similar size to the pAT153 1^m control which implied that these possessed the 81bp ColE1 fragment in the B-lactamase gene. A confirmatory Sau3A digest was performed since two Sau3A sites span the Pst1 site in the bla gene (Fig. 3.6A). The presence of the smallest ColE1 fragment in the Pst1 site should produce a 422bp Sau3A fragment instead of the 341bp fragment found in wild-type pAT153. Figure 3.6A confirms that the 81bp fragment from ColE1 has been inserted at the Pst1 site thereby inactivating the pAT153 bla gene (pCJ104).

3.3.2 CONSTRUCTION OF Ap^rTc^s PAT153 DNA.

To facilitate the screening procedure for dimeric pAT153 molecules containing only one functional bla gene, a Tc^s pAT153 derivative was also constructed. Wild-type pAT153 was restricted with BamHI which cuts the plasmid once in the tetracycline resistance gene. ColE1 DNA was restricted with Sau3A to produce a range of compatible fragments many of which are less than 100bp. Ligation and subsequent transformation of DS903 resulted in the isolation of Ap^rTc^s colonies. The size of insert was estimated by analysing the HaeII restriction pattern since two HaeII sites span the BamHI site. Figure 3.6B shows the HaeII restriction pattern for the Tc^s pAT153 and for wild-type pAT153. The 181bp fragment spanning the BamHI site in the wild-type plasmid has been replaced by a fragment of approximately 235bp indicating that a 56bp Sau3A fragment from ColE1 was inserted in the tetracycline resistance gene at the BamHI site (pCJ103).

To construct the single functional bla multimers, both DNA's were restricted with EcoRI and the Ap^rTc^s DNA was treated with bacterial alkaline phosphatase to minimise self ligation. DS903 was transformed with the ligated DNA and any Ap^rTc^r colonies arising should have been produced by ligation of the two DNAs to produce plasmid multimers. Several Ap^rTc^r colonies were obtained and analysed on single colony gels. Most of the isolates contained plasmid dimers (pCJ108) but some contained plasmid trimers (pCJ109). A schematic diagram showing the construction of these multimers is shown in Fig.3.7.

Figure 3.7

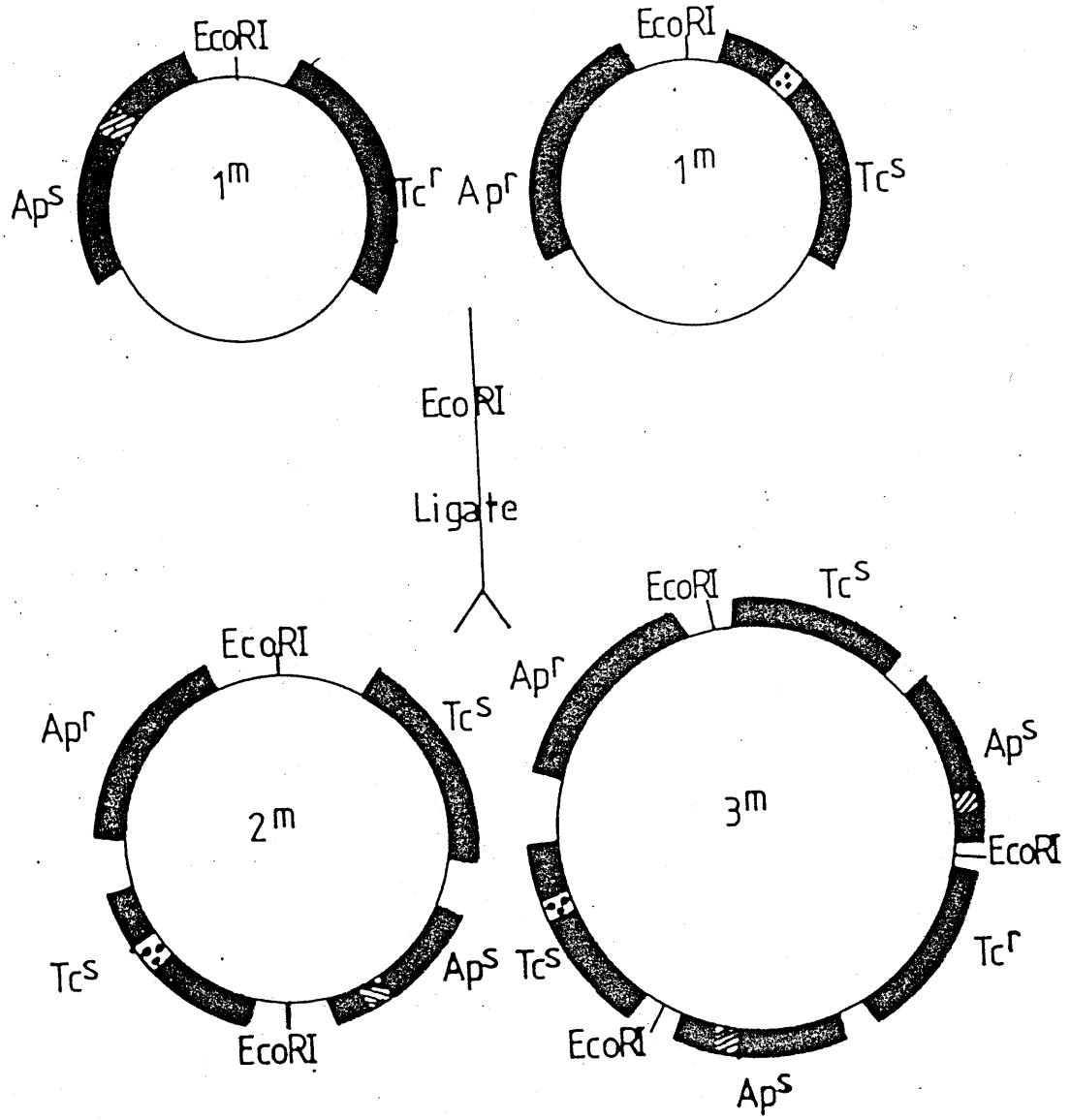


Figure 3.7 A Schematic Drawing of the Construction of Multimers Carrying only One Funtional bla Gene.

Figure 3.8

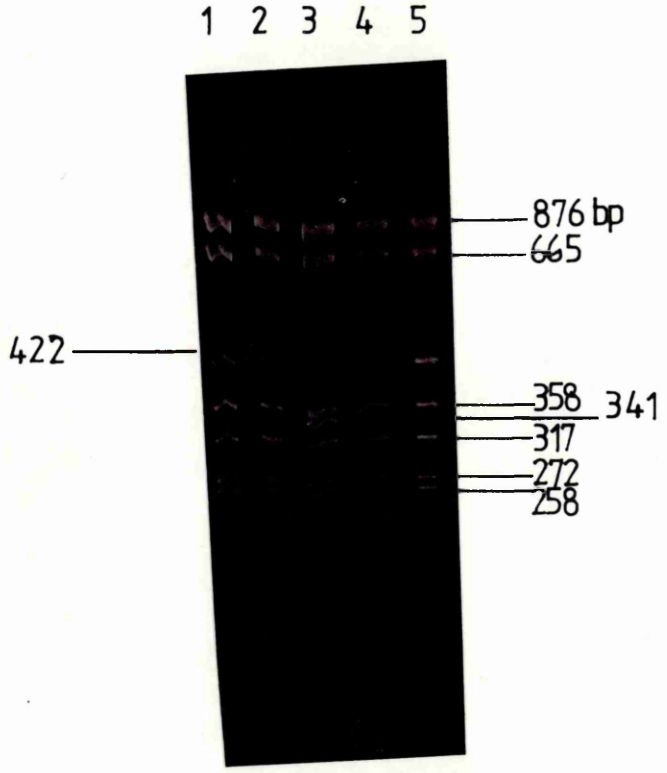


Figure 3.8 Sau3A Restriction Analysis of Multimers Possessing One Functional *bla* gene. 5 % acrylamide gel showing the Sau3A profiles of pCJ109, wild-type pAT153, pCJ108 and pCJ104. Tracks 1 and 2 show that the intensities of the 341 and 422bp Sau3A fragments differ in pCJ109 but are similar in pCJ108.

(1) pCJ109 Sau3A
(2) PCJ109 Sau3A
(3) pAT153 Sau3A
(4) pCJ108 Sau3A
(5) pCJ104 Sau3A

3.3.3. AN ANALYSIS OF THE COMPOSITION OF THE CONSTRUCTED MULTIMERS.

Restriction digests which allowed the relative intensities of the bands corresponding to the Ap^s and the Ap^r genes to be estimated were carried out. The 81bp insert into the Ap^r gene of pAT153 resulted in the Sau3A fragment spanning the Pst1 site to increase from 422bp to 341bp. Figure 3.8 is a 5% acrylamide gel showing the Sau3A restriction patterns of wild-type pAT153, Ap^s pAT153 (pCJ104) and of the multimeric forms of the plasmid. Track 3 shows the restriction pattern of the wild-type plasmid and demonstrates the presence of the 341bp fragment. Track 5 shows the pattern obtained with the Ap^sTc^r plasmid and demonstrates the absence of the 341bp fragment and the presence instead of the 422bp fragment. Tracks 1,2 and 4 show the restriction patterns of the plasmid multimers and demonstrate the presence of both the 341 and the 422bp fragments.

The relative intensities of the two fragments of interest 341 and 422bp in the dimeric plasmid DNA are approximately equivalent whilst the intensity of the 422 band in the trimeric DNA is greater than the 341bp band.

This strongly implied that the 2^m plasmid pCJ108 contained one Ap^s gene and one Ap^r gene while the 3^m plasmid pCJ109 contained two Ap^s genes and one Ap^r gene. Further confirmation was obtained from a transformation assay. pCJ109 DNA and natural 3^m pAT153 DNA were separately restricted with EcoRI, self-ligated and used to transform DS903. If the interpretation of the restriction data is correct then pCJ109 should produce monomeric plasmids resistant to either Ap or Tet in the ratio 1:2. Equal volumes of the transformed cells were plated onto ampicillin and tetracycline- containing plates. Each colony arising was subsequently patched onto an Ap,Tc containing plate to determine how many of the colonies resulted from cells containing multimeric plasmid species. Table 3.1 shows the results of the transformation assay. pCJ109 upon restriction/ligation produced a ratio of Ap^r:Tc^r of 1:2, whilst a natural pAT153 3^m produced equal proportions of colonies which were all both Ap^r and Tc^r.

3.3.4 CONSTRUCTION OF Ap^sTc^r AND Ap^rTc^s PBR322 DNA

Having constructed pAT153 2^m and 3^m molecules possessing one functional ampicillin resistance gene, it was then necessary to produce similar multimers for pBR322.

Plasmid DNA	Ap^r	Tc^r	Ap^rTc^r	Ratio Ap^r : Tc^r
pCJ109	138	274	18,12	Approx 1:2
pAT153 3^m	97	108	All	1:1
pCJ111	112	210	14,19	1:2
pBR322 3^m	123	115	All	1:1

Table 3.1 The Ratio of Ap^r : Tc^r Colonies Produced from Transformation with EcoRI Restricted/Ligated pCJ109, pAT153 3^m, pCJ111 and pBR322 3^m DNA.

Figure 3.9

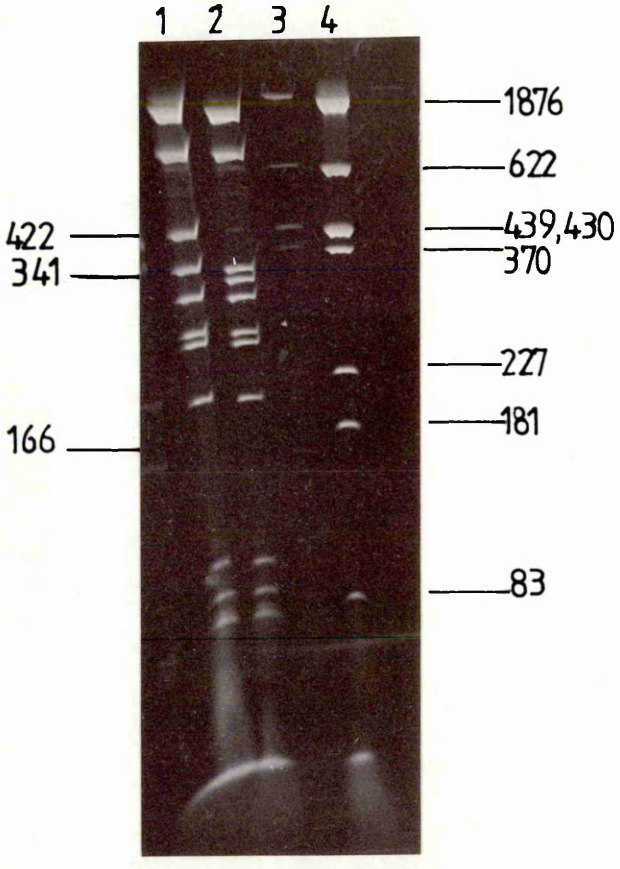


Figure 3.9 Restriction Analysis of pCJ105 and pCJ106. 5% Acrylamide gel of Sau3A restricted pCJ105 and wild-type pBR322 demonstrating that an 81bp fragment from ColE1 has been cloned into the unique Pst1 site in pCJ105 bla producing a Sau3A fragment of 422 instead of 341bp. HaeII restriction analysis of pCJ106 and wild-type pBR322 demonstrates a fortuitous deletion of approximately 15bp in the former plasmid around the BamH1 which renders this plasmid Tc^S.

(1) pCJ105 Sau3A
(2) pBR322 Sau3A
(3) pCJ106 HaeII
(4) pBR322 HaeII

$Ap^{S}Tc^{r}$ pBR322 DNA (pCJ105) was produced using the same method used for pAT153. Restriction analysis of the plasmid revealed that the bla gene had been inactivated by the insertion of the 81bp PstI restriction fragment from ColE1. $Ap^{r}Tc^{S}$ pBR322 (pCJ106) was produced by restricting with BamHI, whereupon a fortuitous deletion of approximately 15bp occurred (Fig.3.9). Plasmid multimers were constructed from $Ap^{r}Tc^{S}$ and $Ap^{S}Tc^{r}$ DNA by EcoRI restriction/ligation and selection for $Ap^{r}Tc^{r}$ colonies after transformation into DS903. Dimeric (pCJ110) and trimeric (pCJ111) isolates were detected by single colony gels and were restricted to determine the number of functional Ap^{r} genes (Fig. 3.10). Once again, the relative intensities of the 341 and 422bp bands demonstrated that the trimeric plasmid was composed of 1:2 Ap^{r}:Ap^{S} genes and the results of the transformation assay confirmed this (Table 3.1). The intensities of the relevant bands in the dimer plasmid were similar, indicating the expected ratio Ap^{r}:Ap^{S} of 1:1.

3.4 Copy Number Determination of Plasmid Multimers Possessing One Functional B-lactamase Gene.

Having obtained pAT153 2^{m}, 3^{m} and pBR322 2^{m} and 3^{m} species with one functional ampicillin resistance gene, it was now possible to determine more conclusively whether the multimeric forms were maintained at lower copy numbers than monomers using single cell resistance to cephaloridine. The plasmid containing cells were diluted and spread onto plates containing increasing concentrations of cephaloridine. Many experiments of this type were carried out and although the actual resistance levels frequently differed, the trends were always similar. Figure 3.11 shows typical survival curves for each strain. It is clear that the level of resistance exhibited by the cells depended upon the multimeric state of the plasmid. The LD_{50} values for pAT153 1^{m}, pCJ105, pCJ106 were 50, 28 and 24ug/ml respectively and for pBR322 1^{m}, pCJ110, pCJ111 47, 26 and 22ug/ml respectively. These results demonstrate that the copy number of the multimeric plasmids is lower than the monomeric form. The resistance of both 2^{m} plasmids (at LD_{50}) was approximately half that of the 1^{m} in each case, although the resistance of the 3^{m} species, was around 47% of the 1^{m} value.

Figure 3.10

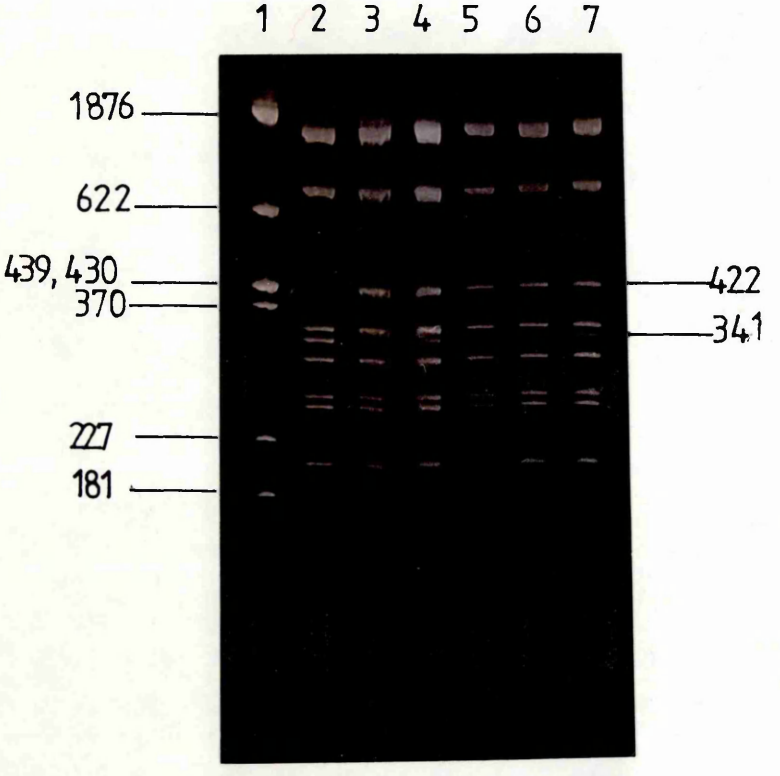


Figure 3.10 Sau3A Restriction Profile of pCJ111. 5% Acrylamide gel showing the Sau3A restriction pattern of pCJ111. The molar ratios of the 341 and 422bp fragments is indicated by the different intensities of each band.

(1) pBR322 HaeII
(2) pBR322 Sau3A
(3) pCJ105 Sau3A
(4) pCJ106 Sau3A
(5) " "
(6) " "
(7) " "

Figure 3.11

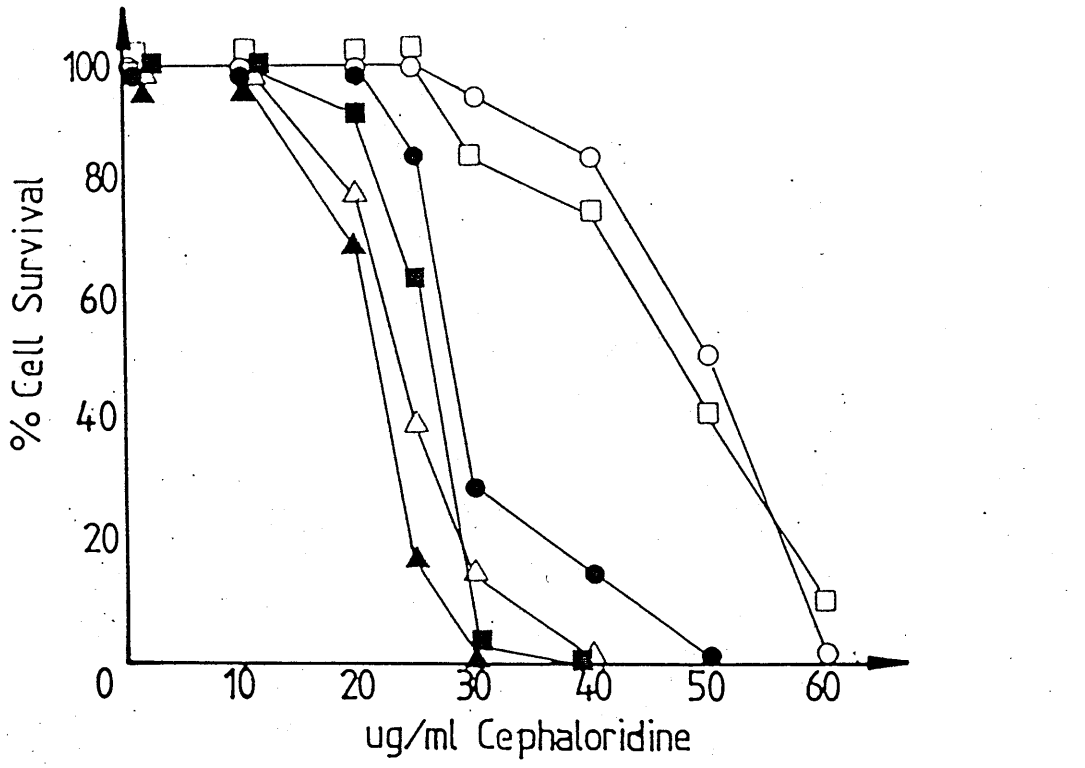


Figure 3.11 Single Cell Resistance to Cephaloridine of pAT153 1^m, pCJ108, pCJ109, pBR322 1^m, pCJ110 and pCJ111. The LD_{50} values for each plamsid containing strain are shown below.

DS903	pAT153 1^m	50 ug/ml cephaloridine
"	pCJ108	28 " " "
"	pCJ109	24 " " "
"	pBR322 1^m	47 " " "
"	pCJ110	26 " " "
"	pCJ111	22 " " "

Strain	Plasmid	Plasmid Size (Kb)	Calculated Copy Number	% Standard Deviation
			(a) (b) (c)	
DS903	pAT153 1^m	3.65	23.0 +/- 3.77 (7)	16
"	pAT153 2^m	7.35	15.0 +/- 1.95 (13)	18
"	pAT153 3^m	10.97	11.0 +/- 2.10 (7)	23
"	pBR322 1^m	4.36	20.0 +/- 3.70 (6)	18
"	pBR322 2^m	8.73	12.0 +/- 2.16 (8)	18
"	pBR322 3^m	13.01	8.0 +/- 1.77 (8)	22
"	pCJ121 1^m	9.99	30.0 +/- 1.87 (3)	6

Table 3.2 Calculated Copy Numbers from *in vivo* Labelling Data. Plasmid copy numbers were calculated per genome equivalent based on a figure of 3.8×10^3kb for the size of a non-replicating *E.coli* chromosome. The calculated copy numbers per genome equivalent are given in column (a), the standard deviations in column (b) and the number of independent trials in column (c). The copy number calculation was as follows:

$$\text{Copy Number} = \frac{\dfrac{\text{cpm plasmid}}{\text{cpm chromosome}} \times \text{Size } \textit{E.coli} \text{ Chromosome (kb)}}{\text{Size of Plasmid (kb)}}$$

3.5 A Quantitative Approach To Determine Plasmid Copy Number.

Since cephaloridine resistance provides an indirect estimate of copy number, a more direct method was chosen to assess copy number in a quantitative manner which relied upon agarose gel electrophoresis to separate plasmid ccc, oc and chromosome. This method is more reliable than CsCl ethidium bromide density gradient centrifugation because it detects open circular plasmid forms, which are not detectable in the plasmid specific band in density gradients.

Plasmid containing cells were grown to exponential phase prior to the addition of ^{3}H thymidine. Deoxyadenosine was added to provide a source of deoxyribose 1-phosphate since the cells were thy^{+}. Thymine prototrophs have a negligible pool of this compound which is required to re-convert thymine to thymidine subsequent to enzyme activity within the cell breaking down the incoming ^{3}H thymidine (Kornberg 1980).

Total DNA was isolated from the labelled cells and aliquots run on 0.8% agarose gels to separate the chromosome and various plasmid forms. After staining with ethidium bromide, the chromosomal and plasmid ccc and oc forms were excised, melted and the incorporated radioactivity estimated in a scintillation counter.

It was necessary to carry out a control experiment to clarify the following points: (1) Is plasmid DNA trapped in the material which remains at the well origin? (2) Is plasmid DNA trapped within the chromosomal bands in the gel?

To answer these points, Southern Blot analysis was performed with a 0.8% agarose gel containing total lysates from pAT153 1^{m}, 2^{m} and 3^{m} and pBR322 1^{m}, 2^{m} and 3^{m} containing strains (Fig 3.12A) probed with linearized, ^{32}P nick translated pAT153 DNA. This gave a representative plasmid size range of between 3.7-13kb. Figure 3.12B, the Southern Blot autoradiograph demonstrates the absence of plasmid trapping at the well origin or within the chromosomal bands.

Using this technique, copy number estimations for pAT153 and pBR322 multimers were obtained (Table 3.2). The copy number of the pBR322 2^{m} is approximately one half of the 1^{m} plasmid, while pBR322 3^{m} is maintained at a level slightly greater than one third of the plasmid 1^{m}. This result lends credence to the cephaloridine resistance data which also implied that the copy number of the 3^{m} plasmids was greater than one third of the monomer.

The *in vivo* labelling experiments, like the cephaloridine resistance data, fail to demonstrate a large difference between the copy number of pAT153 and pBR322 either at the monomeric or multimeric level, although a considerable copy number difference is normally observed between *rom*$^+$ and *rom*$^-$ plasmids (Twigg and Sherratt 1980; Chapter 6 this thesis).

3.6 Does Plasmid Size Influence Plasmid Copy Number?

The results in the previous section demonstrated that the copy number of plasmids was progressively reduced with an increse in the multimeric state of the plasmid. It could be argued that size may affect plasmid copy number if a mass control system rather than an origin counting mechanism existed for copy number control.

Investigating the possibility that plasmid size rather than origin number affects the copy number of the plasmid multimers, required the construction of a single origin plasmid which was the size of a plasmid dimer.

Before cloning large fragments into pBR322 to create such a plasmid, it was first necessary to consider the effect on copy number control that cloning DNA into particular regions of this plasmid would have. Stueber and Bujard (1982) demonstrated that the transcriptional environment surrounding the replication origin of ColE1-like plasmids can affect plasmid copy number, since transcriptional readthrough into the replication region can negatively interfere with plasmid replication. In contrast, transcriptional readthrough in the direction of plasmid replication may enhance replication. The existence of a strong promoter (anti-tet) in pBR322, reading away from the tetracycline resistance gene was demonstrated by Stueber and Bujard (1981). This promoter was found to be 1.5x as strong as the tetracycline resistance promoter and contributed to the expression of the ampicillin resistance gene in this vector (Fig.3.1A; Stueber and Bujard 1981; Von Gabain *et al* 1983).

It is possible that transcriptional readthrough from the "anti-tet" plus the *bla* promoters increases the rate of pre-primer formation (see Chapt.6 concluding remarks) and hence increases plasmid copy number. Indeed, an increase in copy number as a result of readthrough from the *bla* promoter in Ap^r ColE1 derivatives has been demonstrated by Schmidt and Inselburg (1981). The insertion of a large DNA fragment into the Ap^r

Figure 3.13A

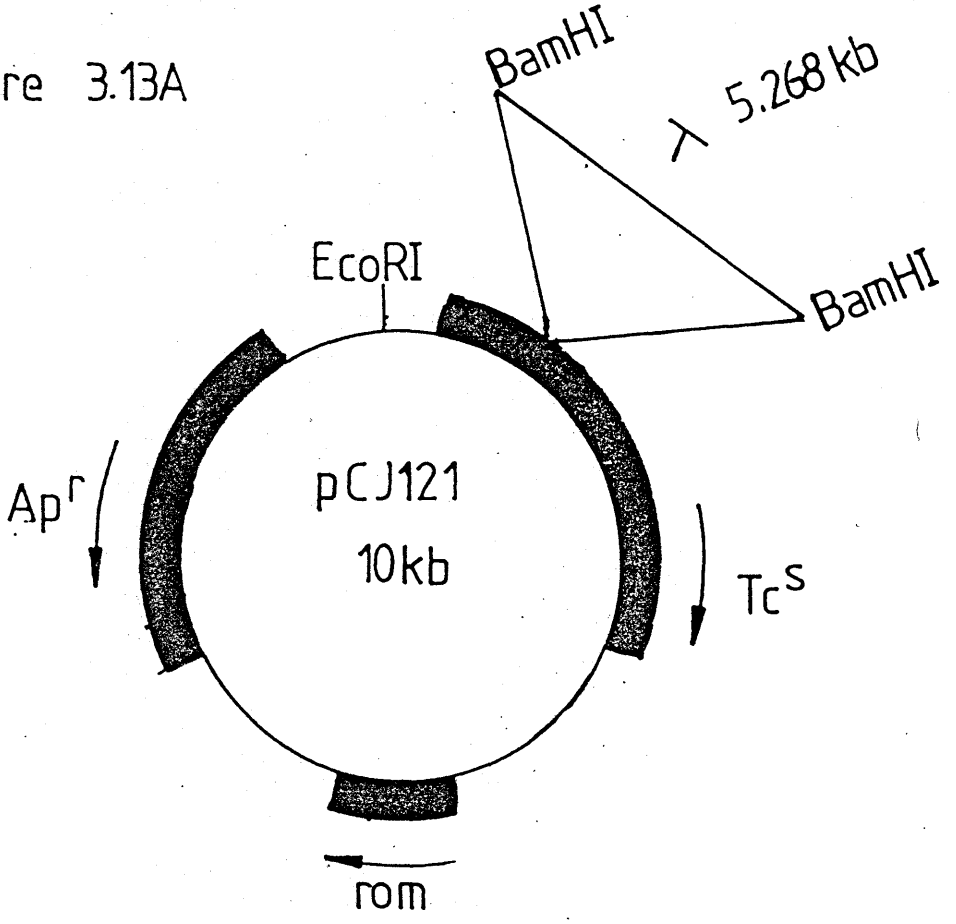
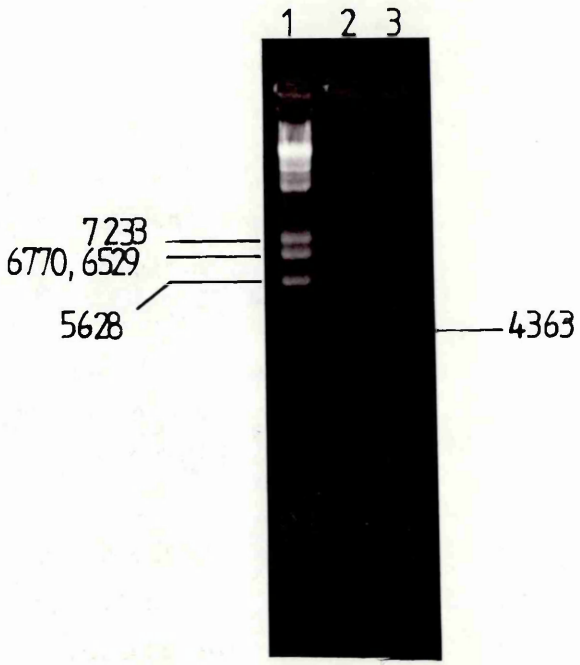


Figure 3.13A Schematic Diagram of pCJ121. This shows the site of insertion of the 5.628kb Lambda insert into the tetracycline resistance gene which is therefore inactivated.

Figure 3.13B



3.13B 1% Agarose Gel Showing the Structure of pCJ121. A BamHI digest of pBR322, pCJ121 and Lambda confirmed the presence of the 5.268kb Lambda fragment in the unique BamHI site in pBR322.

(1) Lambda BamHI
(2) pCJ121 BamHI
(3) pBR322 BamHI

gene reduced the copy number of the plasmid to a value similar to the naturally occurring ColE1 plasmid.

Constructing a one origin, dimer sized plasmid involved cloning a large fragment of lambda DNA into the tetracycline resistance gene of pBR322 so that the regions involved directly or indirectly in copy number control such as bla, "anti-tet" and rom were not disrupted. pBR322 DNA was linearised with BamHI and ligated to BamHI restricted lambda DNA. DS903 was then transformed to ampicillin resistance and the colonies screened for tetracycline sensitivity. Single colony gel analysis revealed that one of the Ap^rTc^s clones appeared to be slightly larger than the size of a pBR322 dimer. Subsequent restriction analysis revealed that the 5628bp fragment from lambda had been cloned into the unique BamHI site, producing a plasmid of around 10kb (Fig 3.13A and B). The 5628bp lambda fragment was derived from the "silent b" region of the phage and therefore was considered unlikely to interefere with the biology of this plasmid construct other than by dint of its size.

The copy number of this plasmid (pCJ121) was estimated by the in vivo labelling technique and from three independant trials was found to be slightly higher than wild-type pBR322 at 30 copies rather than 20 per genome equivalent (Table 3.2). This result indicates that despite pCJ121 being larger than a wild-type dimer, the copy number is not correspondingly reduced to half that of the monomeric plasmid. This implies that the number of plasmid origins rather than plasmid size is the important factor in determining plasmid copy number. The increase in copy number observed for pCJ121 could be due to a reduction in transcriptional readthrough from the tetracycline resistance region into rom. This gene codes for a 63a.a protein which catalyses the binding of RNAI to RNAII and thereby enhances the negative regulatory effect of RNAI on copy number (Tomizawa and Som 1984).

The effect of both origin number and plasmid size on copy number has also been investigated by Summers and Sherratt (1984) using a set of related plasmids, which were not multimers but possessed 1-4 origins of replication. The copy numbers of these plasmids decreased as the number of replication origins increased, while a control plasmid with one origin and a 4kb lambda DNA insert had a copy number not dissimilar to that of the one origin parental plasmid.

Muesing and Polisky (1981) investigated the possibility that plasmid mass could be an important parameter in replication control for ColE1

and its derivatives. Their copy number estimations demonstrated unequivocally that mass control is not an important consideration in ColE1 copy number control. It was also concluded that on the occasions when copy number does appear to be related to plasmid size, plasmid specific negative regulation has been abolished and copy number then becomes controlled by limiting host factors. Consequently, in situations like this, large plasmids will have lower copy numbers than smaller ones simply because some cellular component for DNA chain elongation becomes limiting.

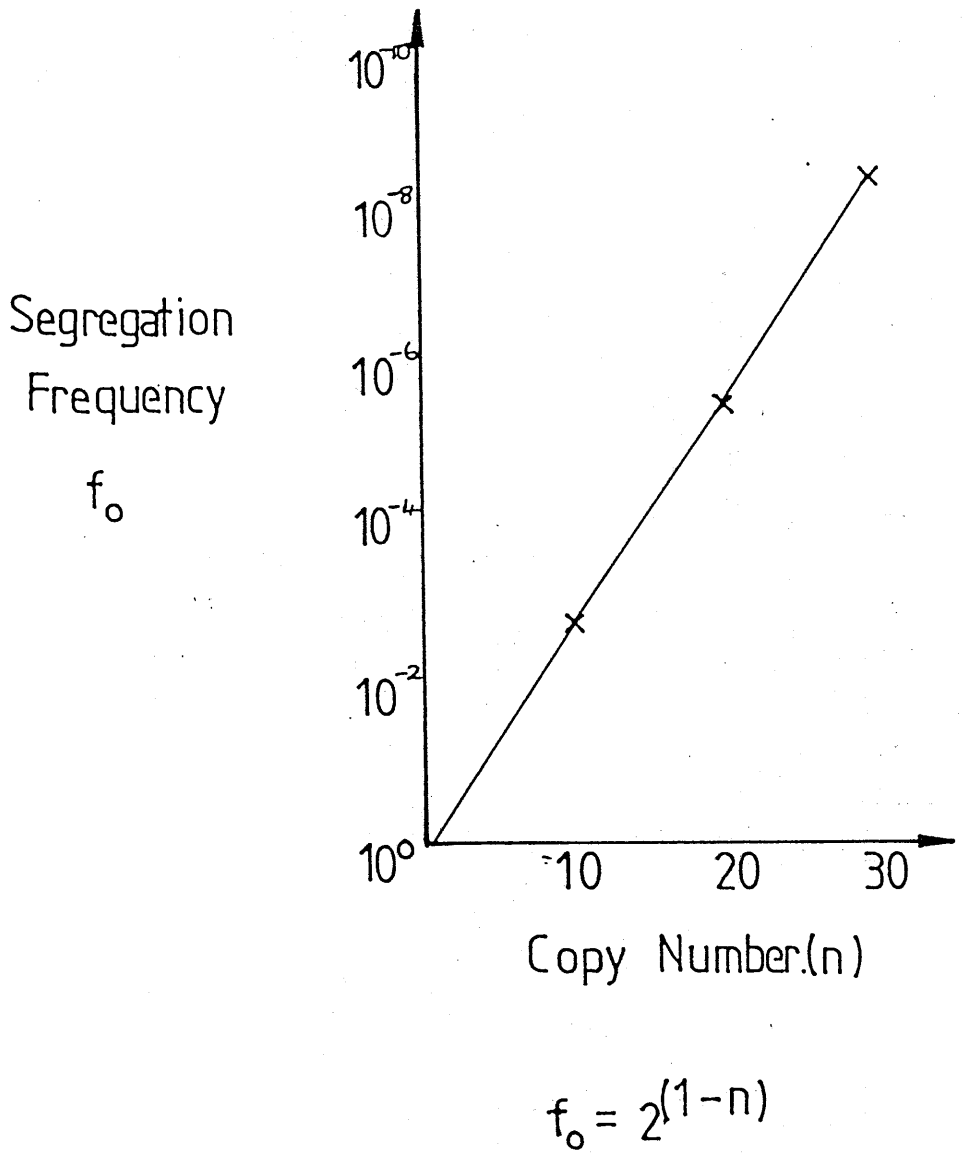
3.7 The Stability of pAT153 and pBR322 Multimers.

The introduction to this chapter briefly mentioned the criteria which must be met for stable maintainance of plasmids. Replication is one obviously important aspect of plasmid maintenance and much is now known about the replication control mechanisms existing for ColE1 and its derivatives.

Although cis-acting regions which promote active partitioning have been found for low copy number plasmids (Meacock and Cohen 1979; Nordstrom et al 1980), no analogous regions have been demonstrated for high copy number plasmids. Several models have been proposed for partitioning of multicopy plasmids including totally random partitioning (Durkacz and Sherratt 1973); active partitioning of all plasmid molecules (Della Latta et al 1978) and a model involving elements of both random and active partitioning at cell division (Hashimoto-Gotoh and Ishii 1982). All of these models are based upon the kinetics of plasmid curing using temperature-sensitive plasmid replication mutants or $PolA_{ts}$ chromosomal lesions as methods to switch off plasmid replication in order to study plasmid segregation.

The partitioning mechanism of ColE1 and its derivatives is unclear but it is probable that they may rely upon random partitioning alone to ensure that daughter cells inherit a plasmid copy. Random partitioning will result in an equal probability of any one plasmid copy going to either of the two daughter cells at cell division and if there are 2n copies per cell, the daughter cells will receive any number between 0 and 2n plasmids. Consequently, plasmid free cells will be generated at a frequency which is related to the copy number of the plasmid at cell

Figure 3.14



$$f_o = 2^{(1-n)}$$

Figure 3.14 Copy Number and Segregation Frequency of Randomly Partitioned Plamsids. This relationship is derived from the binomial distribution and may be expressed mathematically as: $f_o = 2^{(1-n)}$, where "f_o" is the frequency of plasmid-free cell production (per cell per generation) and "n" is the copy number of the dividing cell. From this it is readily seen that the higher the copy number the lower the probability of plasmid-free segregants being produced (After Summers and Sherratt 1984).

division.

The relationship between copy number and segregation frequency is derived from the binomial distribution (Novick et al 1974) and can be expressed mathematically as :

$$f_o = 2^{(1-n)}$$

Where "f_o" is the segregation frequency per cell per generation and "n" is the number of segregating units per cell at cell division. Figure 3.14 shows a graph of segregation frequency versus copy number and demonstrates that the greater the number of segregating units the less likely it is that a plasmid free segregant will arise.

The nature of stability experiments means that segregation frequencies of $>10^{-5}$ can be detected. Using the above expression, copy number per cell at cell division required for a segregation frequency of 10^{-5} per cell per generation was calculated to be 18. Therefore cells at division containing plasmids with a copy number of 18 or greater, would be expected to be stably maintained using this stability assay. Conversely, p^- segregants should be detected when plasmids are maintained at copy numbers less than 18.

Implicit in this are two assumptions:

(1).The plasmid copies within cells are independent segregating units.
(2).All plasmid copies are available for segregation i.e. no plasmid sequestration has occurred.

The stability of pAT153 and pBR322 monomers and multimers was determined in DS903 (Fig.3.15). After 100 generations of non-selective growth both pAT153 1^m and pBR322 1^m were completely stable, which implies a segregation frequency of less than 10^{-5} per cell per generation. The multimeric forms of both plasmids however, were not stably maintained and the calculated mean segregation frequencies over 100 generations for these plasmids were as follows:

pAT153 2^m	7.8×10^{-3}	per cell per generation.
pAT153 3^m	2.0×10^{-2}	" " " "
pBR322 2^m	9.4×10^{-3}	" " " "
pBR322 3^m	2.2×10^{-2}	" " " "

Figure 3.15

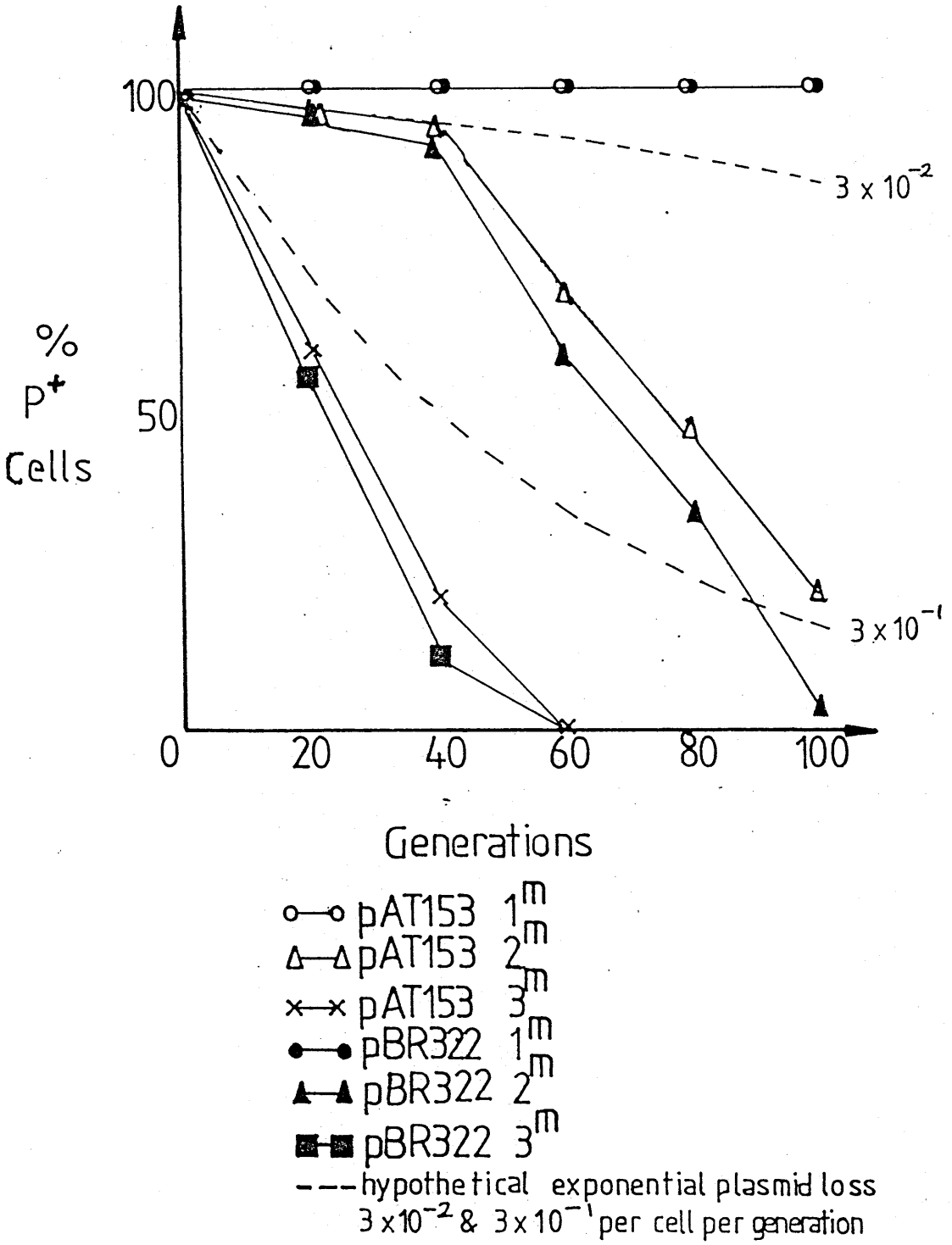


Figure 3.15 Stability of pAT153 1^m, 2^m, 3^m and pBR322 1^m, 2^m and 3^m in DS903. The % of plasmid containing cells in batch culture was measured every 20 generations for a total of 100 generations and this graph shows the typical stability profiles of these plasmids. Their mean segregation frequencies over 100 generations are listed in section 3.7. Two theoretical plots representing segregation frequencies of 3×10^{-2} and 3×10^{-1} per cell per generation over 100 generations are also shown and their relevence is discussed in section 3.9.5.

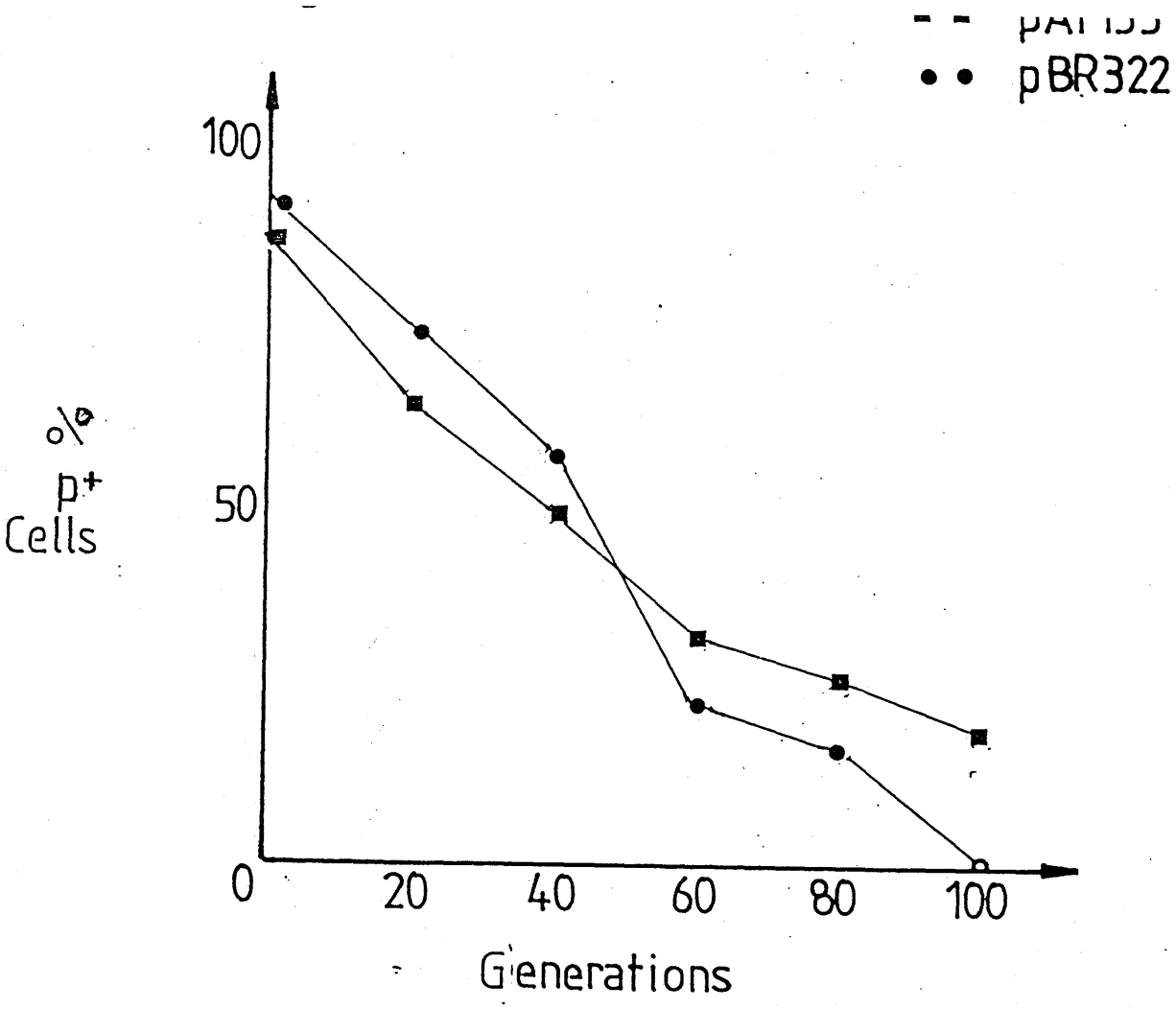


Figure 3.16 Stability of pAT153 and pBR322 in JC8679. The % of plasmid-containing cells was measured every 20 generations over 100 generations. Both pAT153 and pBR322 are unstable in this strain with mean segregation frequencies over 100 generations of 6.7×10^{-3} and 7.6×10^{-3} per cell per generation respectively.

Clearly the trimeric forms of both plasmids are less stably maintained than the dimeric forms and both are less stable than the monomeric species.

If plasmid instability results from a reduction in the number of segregating units due to multimerization, these plasmids should also be unstable in the hyper-recombinogenic strain JC8679. Typical stability graphs for both pAT153 and pBR322 in this strain are shown in figure 3.16 and both plasmids are indeed unstable in this strain. These plasmids can be stably maintained in JC8679 when cer, the ColE1 site at which site-specific recombination acts to resolve multimers to monomers is cloned into these vectors (Summers and Sherratt 1984).

3.8 Can the Calculated Copy Numbers for pAT153 and pBR322 1^{m} 2^{m} and 3^{m} Account for the Observed Instabilities of These Plasmids?

Table 3.2 shows the calculated average plasmid copy numbers per genome equivalent in a bacterial population. For the purpose of calculating segregation frequencies however, plasmid copy number per genome equivalent is insufficient, since information on the copy number per dividing cell is required and it cannot be assumed that dividing cells possess only 2 chromosomes.

Cooper and Helmstetter (1968) proposed a model to explain the observation that the amount of DNA per cell varies with growth rate. Their experimental data demonstrated that the transit time of replication forks for cells with doubling times less than 70 minutes was almost constant in E.coliB/r. This indicated that the frequency of initiation was the factor which could account for varying DNA content with doubling time. The time required to replicate one chromosome was around 40 minutes and cell division did not occur until 20 minutes after termination of chromosome replication. On this basis they predicted that multiple rounds of replication initiation must occur in cells with doubling times faster than 40 minutes.

The generation times of the plasmid containing cells during stability and copy number experiments were similar, around 30 minutes. According to the Cooper Helmstetter model, cells with a doubling time of this order will possess approximately 3.75 genome equivalents per dividing cell (Fig.3.17).

Figure 3.17

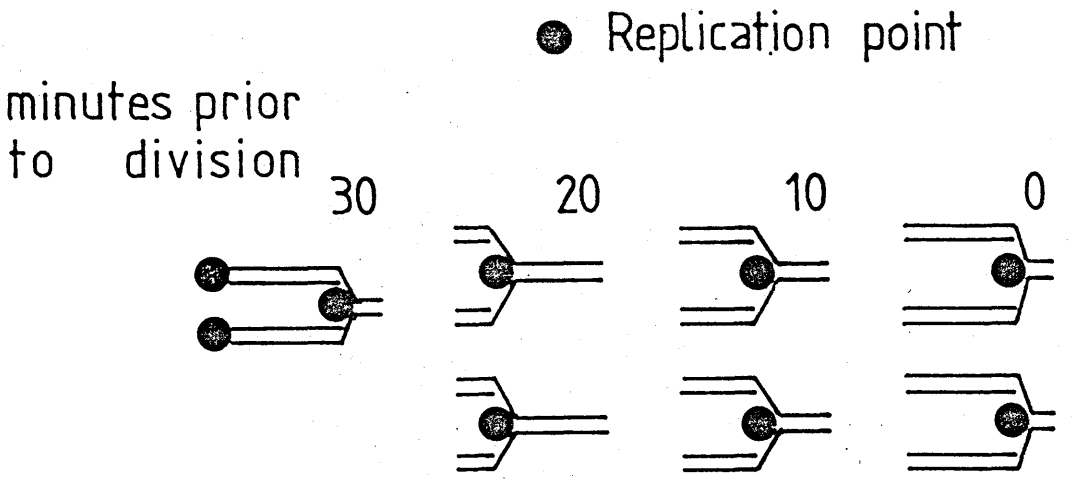


Figure 3.17 Chromosome Configurations of Cells with a Doubling Time of 30 minutes. Since time taken for one replication fork to traverse the E.coli chromosome was calculated to be 40 minutes (Cooper and Helmstetter 1968), cells doubling every 30 minutes must possess more than one replication point in order to replicate the required complement of chromosomes prior to cell division. The time between the end of chromosome replication and cell division was calculated to be 20 minutes (Cooper and Helmstetter 1968). Thus cells with a doubling time of 30 minutes have just 30-20 = 10 minutes, in which to produce a minimum of 2 genome equivalents. Since complete chromosome replication takes 40 minutes, after 10 minutes, only 0.25 of a chromosome will have been replicated. Therefore, at cell division each daughter cell must receive 1.75 chromosomes and 20 minutes prior to the following cell division there will be a minimum of 2 chromosome equivalents. At cell division there will be approximately 3.75 genome equivalents per cell.

Strain	Plasmid	C.N. per genome equivalent	C.N. per Dividing Cell	Segregation Frequency.
DS903	pAT153 1^m	23	86	$2x10^{-26}$
DS903	pAT153 2^m	15	56	$3x10^{-17}$
DS903	pAT153 3^m	11	41	$9x10^{-13}$
DS903	pBR322 1^m	20	75	$5x10^{-23}$
DS903	pBR322 2^m	12	45	$5x10^{-14}$
DS903	pBR322 3^m	8	30	$2x10^{-9}$

Table 3.3 Calculated Copy Number per Dividing Cell Assuming 3.75 Chromosome Equivalents at Division. The copy number values from table 3.2 were multiplied by 3.75 in order to estimate the copy numbers and segregation frequencies of pAT153 and pBR322 multimers in dividing cells.

The copy number calculation relies upon the ratio of plasmid specific counts to chromosome counts and involves the size of the E.coli chromosome to calculate the number of copies of a plasmid of known size. Clearly therefore, knowledge of the number of chromosome equivalents per cell is required for accurate estimation of plasmid copy number. Since copy number estimations were carried out on non-synchronous, exponentially growing cells possessing all possible chromosome configurations, the figures in table 3.2 are at best average values. Therefore in order to obtain estimations of copy number at cell division, the figures in table 3.2 were multiplied by 3.75 and the results and calculated segregation frequencies are shown in table 3.3. These segregation frequencies predict that all six plasmids should be very stable but this is evidently not the case. In addition, when a minimum value of 2 chromosome equivalents per dividing cell is assumed, the calculated segregation frequencies also predict greater stability than is observed.

3.9 Possible Explanations for the Observed Plasmid Instability.

There are several ways to explain the discrepancy between the predicted and the observed stability results.

(1). Inaccurate copy number determination.
(2). Plasmids not segregating as individual segregating units or all plasmid copies are not available for segregation due to sequestration.
(3). Variance in plasmid copy number between cells.
(4). Competition.

3.9.1 INACCURATE COPY NUMBER DETERMINATION.

Firstly, it was possible that the copy number determinations were totally inaccurate. This was considered unlikely since in each case a relatively large number of independent trials were carried out. In addition, trapping of chromosomal material in the gel well origin and background counts in the gel tracks were measured during the copy number estimation experiments and the relevant corrections applied. Southern

Blot analysis also demonstrated that plasmid specific material was not trapped in either the well origin of the gel or in the chromosomal bands.

The observed standard deviations (Table 3.2) are similar to those observed by others (Projan *et al* 1983) and are probably due to experimental error, perhaps at the lysate mixing stage prior to loading samples onto agarose gels. Any variation in plasmid copy number between cells cannot be detected by this technique since approximately 10^7 cells are lysed during the proceedure and consequently any copy number variance will be masked by sample size.

3.9.2 PLASMID CLUMPING.

Copy number estimation techniques such as hybridization assays and *in vivo* labelling techniques directly measure the amount of plasmid DNA in relation to the chromosome, but no technique reveals the location within the cell or physical state of the plasmids. If plasmid copies in cells aggregate together in some way, the number of individual segregating units will be reduced. At cell division, random partitioning will result in the production of plasmid free cells, detectable by the stability assay, when the number of segregating units falls below the level required for stable maintainance.

Plasmid clumping could occur due to incorrect events at the termination of replication, or due to some RNA or protein molecule acting as a molecular "glue".

The concept of plasmid clumping is not without precedent. Tucker *et al* (1984) suggested that the severe form of instability exhibited by pSC101 plasmids deleted for the entire *par* region ("super-par") could be explained by plasmids within cells segregating as a single unit. Their conclusion was based upon the observation that *par*$^-$ plasmids, temperature sensitive for replication at 42°C, produced plasmid free cells after one cell doubling. In contrast, when the plasmid was *par*$^+$, plasmid free segregants did not appear until several generations had elapsed. Copy number determinations demonstrated that the copy numbers of both *par*$^-$ and *par*$^+$ plasmids were identical. These data led the authors to speculate that the presence of a *par* sequence allows the individual plasmids to be recognised as individual segregating units.

Sakakibara *et al* (1976) demonstrated the existence of a small

proportion of catenated ColE1 molecules in an *in vitro* replication system which were not converted to monomers. This suggested that they were not the normal precursors of monomeric ColE1 plasmids but that they arose by faulty termination of replication. It is difficult to rationalise why constructed plasmid vectors such as pAT153 and pBR322 should form catenanes with greater frequency than the parental plasmids, since both possess identical replication origins and termini. Also, single colony gel analysis of pAT153 and pBR322 1^m, 2^m and 3^m containing stains show a ccc band with only a faint trace of open circular DNA. A catenane composed of two plasmid units would run in a similar position to a dimeric plasmid molecule (M.Boocock pers.comm.) however no bands running in this position or higher up the gel have been observed for these plasmids in DS903 (*recF*). It therefore seems unlikely that plasmid catenation plays a major role in the instability of pAT153 and pBR322 multimers in DS903.

For plasmid clumping to occur via a "molecular glue" mechanism, presumably a binding site on the DNA molecule is required. The composition of both plasmid vectors is known. Both possess part of the tetracycline resistance region from pSC101, the Ap^r gene from Tn3 and the replication region of pMB9 (Sutcliffe 1978). In addition, pBR322 possesses the region coding for Rom, a *trans-acting* copy number control element. Since most of these regions are obtained from the stably maintained, presumably non-clumping plasmids pMB9 and pSC101, it seems unlikely that any DNA region present on the constructed vectors would act as a site at which plasmid aggregation would occur.

3.9.3 PLASMID SEQUESTRATION.

Plasmid instability could occur if all plasmid copies within a cell were not available for random partitioning at cell division. Copy number estimation techniques are unable to determine the location of plasmids within a cell and it cannot be assumed that plasmids float freely within the cytosol.

Kline and Miller (1975;1976) demonstrated that some plasmids co-sediment with the bacterial chromosome, which inferred that there was an association between host chromosome and episomes. Curiously, unit copy plasmids such as the F factor are found almost totally complexed with

the chromosome (>90%) while only around 8% of ColE1 is chromosomally associated. Copy number effects, plasmid size and artifactual binding were not responsible for the observed plasmid-chromosome association and it has been suggested that the degree of chromosome binding is dependent upon some feature of the individual plasmid species. Binding of F to the chromosome may be an added stability feature of this plasmid which results in accurate partitioning at cell division.

The following data further indicate that plasmid copies are not randomly dispersed within the cell cytoplasm. Firstly, an association between the replication origin of some ColE1 molecules and the cell membrane of E.coli minicells was demonstrated by Sparks and Helinski (1979). Secondly, the question of plasmid sequestration/compartmentalization was invoked to explain the results of Novick et al (1980). They studied plasmid curing during the artificial process of formation and regeneration of protoplasts in the gram positive bacterium S.aureus. Certain plasmids were stably maintained during this process, while others were not. This phenomenon was independent of plasmid size or copy number and was suggested to reflect the different locations within cells of different plasmid species. The non-curable plasmids could have been associated with the chromosome, whilst the curable plasmids may have been compartmentalised within the cells in a region normally divided between the daughter cells during cell division.

In order for the instability of the high copy pAT153 and pBR322 multimers to be explained by sequestration, it is necessary to assume that these vectors have an affinity for some cellullar component which the parental, stably maintained plasmids lack. The origins of the various component parts of the vectors have already been discussed and similar to the plasmid clumping idea, it is not obvious why these vectors should be sequestered. Furthermore, the sequestration and clumping arguements are also weakened by the observation that monomeric forms of the plasmids, identical to the plasmid multimers, are stably maintained. This observation suggests that copy number, the one difference between the plasmid forms, directly influences the stability of these vectors.

Figure 3.18

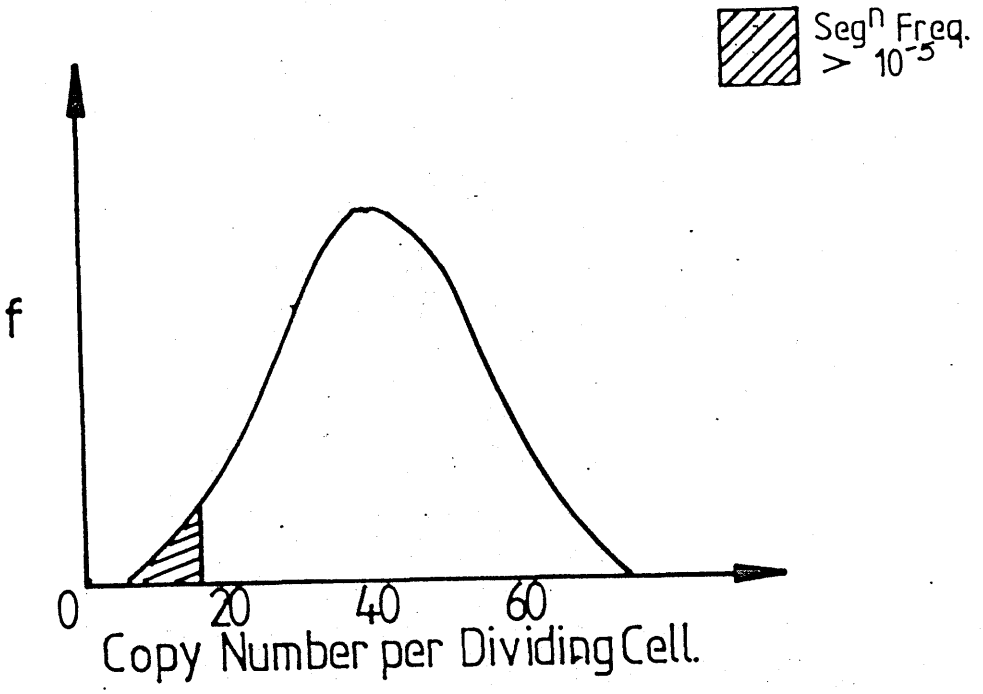


Figure 3.18 Theoretical Distribution of Plasmid Copy Number in a Population of Dividing Cells. Despite a high mean copy number, a plasmid may exhibit copy number variance resulting in some cells in a population possessing low levels of plasmids. These cells may give rise to plasmid-free segregants.

Figure 3.19

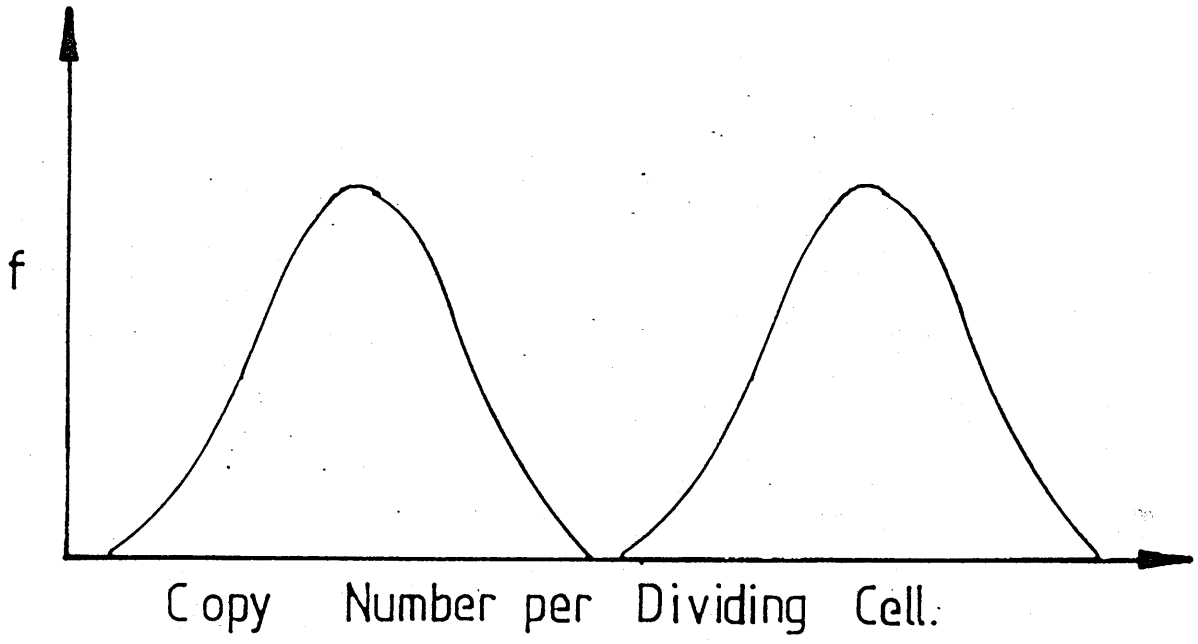


Figure 3.19 Copy Number Variance Could Result in Instability of a High Mean Copy Number Plasmid. Theoretical graph showing the copy number distributions of two high mean copy number plasmids exhibiting the same level of variance. The plasmid with the lower mean copy number which is more than sufficient to ensure hereditary stability, will have a higher probability of generating plasmid-free cells than the higher mean copy number plasmid.

Figure 3.20

```
                5903       5913       5923       5933       5943       5953
COLE1      :GGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTA
            ************************************************************
PBR322     :GGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTA
                2985       2995       3005       3015       3025       3035

                5963       5973       5983       5993       6003       6013
COLE1      :GCTCTTGATCCGGCAAACAAACCACCGTTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC
            *************************** ********************************
PBR322     :GCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC
                3045       3055       3065       3075       3085       3095
```

The sequence of part of the RNAI and RNAII coding regions of ColE1 and pBR322 are shown above. The ColE1 coordinates refer to the sense strand for the rom gene numbered from the unique EcoRI site. The pBR322 coordinates are as given by Sutcliffe (1978). The loss of sequence homology at position 5981 ColE1, 3063 pBR322 results from the inc9 mutation in the latter sequence.

3.9.4 COPY NUMBER VARIANCE.

The prediction from copy number data of segregation frequencies for randomly partitioned plasmids makes the assumption that the copy number is the same in all cells at division. This may not hold for multicopy plasmids. If copy number variance does occur, one could imagine that the distribution of plasmid copy number in a population would follow that shown in Figure 3.18, with cells possessing plasmids at copy numbers above and below the mean copy number. To account for copy number variance in terms of replication control, it is necessary to propose that the control system is rather inefficient at correcting deviations from the mean copy number.

Pritchard (1978) defined an "active" replication control system as one in which deviations from the average copy number are "sensed" and the frequency of replication initiation altered such that the mean value is re-attained. An essential element of a replication control system which can maintain a relatively constant copy number per cell, is a negative feedback loop. This criterion is met in pAT153 and pBR322 by the RNAI/RNAII interaction and in pBR322 by the small *trans*-acting Rom protein which enhances the binding of the two RNA species. The speed with which an inhibitor corrects any copy number deviation will depend upon the stability of the inhibitor, in addition to the nature of the repressors interaction with it s target.

By using various pairs of mutant and wild-type homologous RNAI/RNAII molecules, Tomizawa and Som (1984) investigated the kinetics of interaction of inhibitor with its target. The nucleotide sequence of the RNA species was shown to affect the rate of binding of RNAI to RNAII, such that the wild-type ColE1 RNA species bound more rapidly than several homologous *inc* mutant forms differing by only one base pair. In addition, the 63 residue Rom protein was demonstated to enhance the rate of binding of inhibitor to target, while the efficiency with which this occurred again depended upon the particular RNA molecules.

The replication control regions of both pAT153 and pBR322 are very similar to ColE1, differing by only by 1 base pair (Hashimoto-Gotoh and Timmis 1981; Fig.3.20). The point mutation involved is an A-T to G-C transition at position 3062 (co-ord. Sutcliffe 1978) within the loop region of palindrome 1 of RNAI. This change from the wild-type ColE1 sequence is known as the *inc*9 mutation (Tomizawa and Itoh 1981) and

could be a fundamental difference between the natural pMB1 plasmid, from which the replication origin region of pBR322 is derived and its close relative ColE1. The *in vitro* binding studies of Tomizawa demonstrated that the rate of binding of *inc*9 RNAI to *inc*9 RNAII was half that observed for the "wild-type" RNA species and that the binding enhancement by Rom was less effective with *inc*9 RNA molecules. These data correlated well with *in vivo* copy number estimations for the corresponding plasmids which showed that the *inc*9 plasmid had a higher copy number than the wild-type plasmid.

Since the rate of RNAI/RNAII binding is clearly of great importance for efficient control of replication initiation, it is probable that the lower binding rate of the pBR322 and pAT153 RNA species would result in reduced sensitivity to copy number deviations compared with the stably maintained ColE1 plasmid.

At cell division, assuming random partitioning, plasmid copies will be distributed binomially between daughter cells. Consequently, many of the cells will receive a lower than average number of plasmid copies and similarily a proportion of cells will receive higher than average plasmid copies per newborn cell. If the replication control system cannot correct the downward deviations prior to the next cell division, the cells will give rise to plasmid free segregants.

Employing the variance argument to explain the instability of plasmid multimers, it can be assumed either that the level of copy number variance is equivalent for monomers and multimers, or that multimers exhibit greater variance than plasmid monomers. The second possibility seems unlikely since the replication control system of monomers and multimers are identical. Assuming that the first option is correct, one would predict that the plasmid monomers, despite variance, have a sufficiently high mean copy number to ensure a low probability of cells generating plasmid free segregants. Cells containing multimers with lower mean copy numbers will have a higher probability of producing segregants (Fig. 3.19).

In order to detect copy number variance, it is necessary to determine the plasmid copy number in individual cells; a problem which has prevented a full understanding of the efficiency of replication control systems. The need to reversibly switch off plasmid replication is a basic requirement for estimating the copy number in individual cells in order that continued division of the host cells results in plasmid

copies being diluted out. One attempt to demonstrate copy number variance is discussed in Chapter 6.

3.9.5 COMPETITION.

Stability experiments do not measure solely the segregation frequency of a plasmid, but instead a combination of both segregation and competition between p^+ and any p^- cells which have arisen.

The maintenance cost to cells containing plasmids involves the additional replication of plasmid DNA and synthesis of RNA and protein. It is likely that the metabolic burden imposed on cells containing plasmids will result in them being less "fit" under non-selective growth conditions in comparison to plasmid free cells. Indeed, there are several reports of this in the literature (Godwin et al 1979; Jones et al 1980; Wouters et al 1983; Helling et al 1981).

The data from Chapter 5 demonstrate that in chemostat culture, pAT153 1^m containing cells were at a competitive disadvantage in mixed culture with otherwise isogenic plasmid free cells and therefore it is possible that competition may partly explain the level of instability observed for pAT153 and pBR322 multimers.

Detection of competition between p^- and p^+ cells in mixed culture with unstable plasmids is complicated by the inability to determine the extent to which segregants contribute to the level of p^- cells during the course of the experiment. This problem can be overcome by the construction of differentially marked host strains, so that one can distinguish between plasmid free segregants arising during the experiment and the plasmid free cells added at the start of the competition experiment. Such a strain must have similar growth characteristics to the parental strain from which it is derived in order to avoid the complication of competition between the two plasmid free strains. A DS903 derivative strain which carried a spectinomycin resistance marker was constructed (Chapt.5 and 6) however its growth characteristics were very dissimilar to the parental strain and it was therefore unsuitable for use in competition experiments.

Figure 3.15 shows the stability graphs of pAT153 and pBR322 1^m, 2^m and 3^m forms. If segregation alone occurs one would expect exponential decay curves like the hypothetical ones shown in this figure. The graphs

of the dimeric plasmid species, provide evidence that simple exponential decay kinetics alone cannot account for the shape of the curves. After 20 generations of non-selective growth, 3% of both stains are plasmid free. However, between 40 and 100 generations the segregation frequency seems to increase dramatically and the percentage of plasmid containing cells after 100 generations resembles the end point for the hypothetical $3x10^{-1}$ decay curve. To explain the sudden increase in segregation frequency after 40 generations one would have to assume that the copy number of the plasmids is reduced during the experiment. This seems improbable because the growth conditions were constant throughout all stability experiments.

The discrepancy between the segregation frequencies during the stability experiment may be explained by competition. After 20 generations the 3% plasmid free cells which have arisen, compete with the plasmid containing cells until the combination of further segregants arising and the growth of the "fitter" p^- cells results in rapid take over of the culture by plasmid free cells.

In conclusion, I suggest that both pAT153 and pBR322 are randomly partitioned and the monomeric species is stably maintained because the copy number in all cells is sufficiently high to ensure that segregation frequencies $>10^{-5}$ per cell per generation are not observed. This implies copy numbers of greater than or equal to 18 per cell at cell division. Since the multimeric plasmids are not stably maintained although their estimated mean copy numbers per dividing cell are much greater than required for stable maintainance, this infers that either all copies within a cell are not available for segregation; that the number of segregating units is reduced by clumping or that there is variance in plasmid copy number between individual cells.

The clumping and sequestration arguements seem rather implausible since both vectors have been derived from the same diverse sources (Sutcliffe 1978) and none of these sources exhibit instability.

It is unfortunate that the determination of plasmid copy number in individual cells at cell division is not readily accomplished, since copy number variance seems a more plausible explanation for the observed instability. Variance could result from the inability of the replication control system to respond rapidly to deviations from the mean copy number. Plasmid free cells arising as a result of variance

would have a competitive advantage over plasmid containing cells and would explain the observed high levels of instability of the plasmid multimers.

CONCLUDING REMARKS

(A). REPLICATION AND MAINTENANCE OF MULTICOPY PLASMIDS.

The ColE1 replication control system relies upon a highly specific interaction between the two counter transcripts RNAI and RNAII. Binding of the two RNA species prevents formation of an RNA/DNA duplex at oriV, the substrate for RNAase H processing. The importance of binding rate for efficient negative control was deduced from the observation that Rom protein enhanced the rate of binding between RNAI and RNAII *in vitro* while *in vivo*, *rom*$^+$ plasmids were demonstrated to have lower copy numbers than their *rom*$^-$ counterparts (Tomizawa and Som 1984).

When a plasmid encoded inhibitor is synthesised constitutively and has a short half-life, its rate of synthesis will be proportional to the gene dosage (Nordstrom 1984). Since the inhibitor-target binding reaction of ColE1 occurs via complementary nucleotides and RNA1 has an active half-life of 1-2 minutes (Elbe *et al* 1984) it is probable that there is a 1:1 correspondence. This predicts that there will be a proportional response to deviations in copy number such that a 2 fold increase in copy number will result in a frequency of replication per plasmid of 0.5, while a copy number of 0.5 will result in each plasmid having a frequency of replication of 2.

This type of replication control differs from the control system of F which "senses" a 2 fold difference in copy number and reacts by switching replication on or off. In contrast, the former control system will respond gradually to copy number deviations. This type of control has been proposed for R1, a low copy number plasmid which uses anti-sense RNA to negatively control its copy number. It is possible that the response to copy number deviations need not be rapid for R1 since it possesses a *par* region to ensure faithful segregation. ColE1 lacks a *par* region and is randomly partitioned therefore it may be important that its replication control system can respond quickly to copy number

Figure 3.21

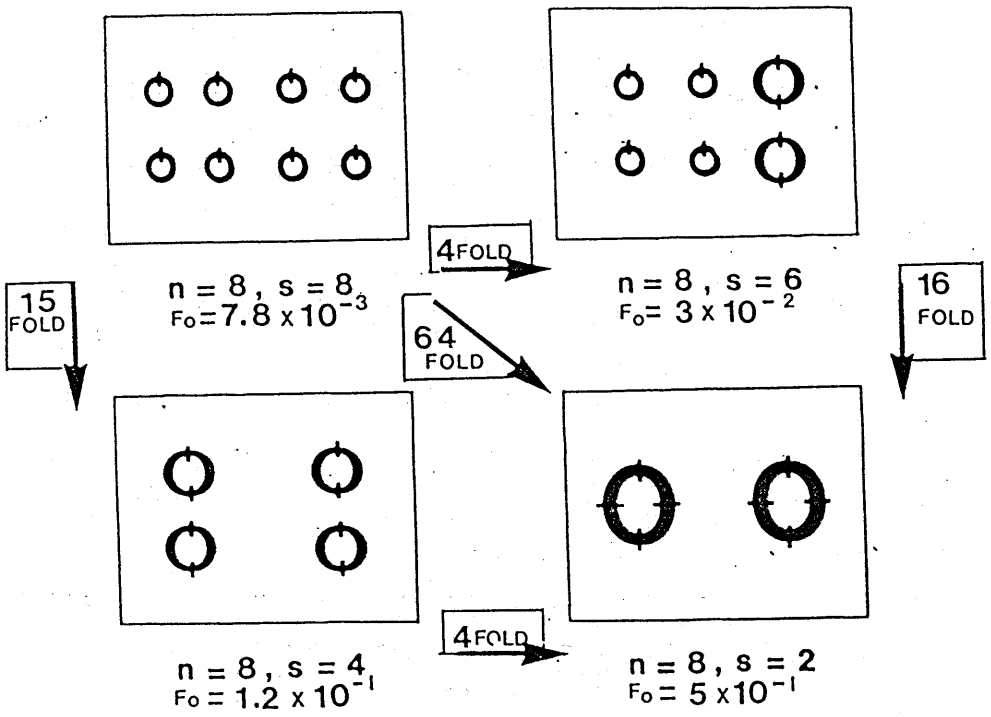


Figure 3.21 Multimerization Reduces the Number of Segregating Units. Schematic diagram showing the effect of multimerization on the number of segregating units. The numbers in the boxes underlined by arrows indicate the effect of multimer formation on the predicted segregation frequencies. "n" = the number of replication origins; "s" = the number of segregating units; f_o = the segregation frequency.

deviations which would restrict copy number variance to a minimum.

It is plausible that the ColE1 Rom protein not only decreases copy number 5-7 fold (Twigg and Sherratt 1980) but in addition may increase the sensitivity of the control system, since *in vitro* the concentration of RNAI required to bind to RNAII is lower in the presence of the Rom protein (Lacatena *et al* 1984). This infers that *in vivo*, in the presence of Rom, small changes in RNAI levels may have a greater effect on the frequency of replication initiation than RNAI alone can produce (see next section).

It has been assumed that the *par*$^-$ ColE1 plasmid is randomly partitioned (Summers and Sherratt 1984). For such a casual system to ensure stable maintainance, plasmid copy number must remain above a threshold value. Variance in copy number could result in plasmid instability but may be kept to a minimum in ColE1 by the action of Rom. This work and work of others (Summers and Sherratt 1984) has demonstrated that plasmid multimerization progressively lowers the number of segregating units and implies that an "origin-counting" mechanism exists for ColE1-type plasmids. Figure 3.21 summarizes how a low level of multimerization reduces the number of segregating units and dramatically affects plasmid segregation frequency.

It has been postulated that multimeric molecules could have a replication advantage over plasmid monomers if there is an equal probability that DNA replication will initiate at any plasmid origin in a cell (Summers and Sherratt 1984). This would result in clones of highly multimerized plasmids within the population which would give rise to plasmid free segregants. In recombinant proficient host strains therefore, multimerization of randomly partitioned plasmids will result in instability. This problem is overcome for ColE1 by *cer*, a 280bp region at which site-specific recombination occurs to resolve multimeric plasmid molecules to monomers (Summers and Sherratt 1984). Sequence analysis demonstrated that the closely related plasmid ColK, possesses a similar region *ckr* which is 69% homologous to *cer* the ColE1 site (Summers *et al* 1985) whilst pMB1, another ColE1 relative, has a region which is almost totally homologous to the *cer* region (Summers and Sherratt 1985).

A similar site-specific recombination site *par*B has been shown to exist in the multicopy CloDF13 plasmid (Hakkaart *et al* 1984) and P1, a unit copy plasmid, encodes a site, *lox*P at which the plasmid encoded *cre*

gene product acts to convert dimeric P1 molecules to monomers (Austin et al 1981). An as yet unidentified host encoded function is thought to be responsible for site-specific recombination between cer sites on ColE1 multimers. The demonstration of site-specific recombination sites on several multicopy plasmids suggests that they may be a common feature to plasmids in this category.

Most natural gram negative plasmids can be transferred between bacteria by conjugal transfer which may be considered to be a stability mechanism. Many large plasmids are self-transmissible and encode all of the gene functions required for transfer. The complexity of the process is reflected by the fact that around one third of F-factor DNA is essential for transfer. Small multicopy plasmids like ColE1 are not self-transmissible but are termed "mobilizable" since they can only be transferred from cell to cell when they are co-resident with a conjugative plasmid (Willetts and Wilkins 1984).

(B) ORIGIN COUNTING -HOW CAN IT BE ACHIEVED?

Several models have been proposed to explain negative control of plasmid copy number. Pritchard's Inhibitor Dilution Model predicted the existence of a plasmid encoded inhibitor which was constitutively produced and had a short half-life. As replication proceeds, the concentration of inhibitor increases until plasmid replication is shut-off. Cell growth leads to dilution of the inhibitor concentration, whereupon plasmid replication initiation can occur. This model however was proposed to explain replication control of unit copy plasmids which exhibit "switch-on/switch-off" replication. Pritchard (1978) considered that his model was unlikely to produce "switch-on/switch-off" type replication for multicopy plasmids, due to fluctuation in the timing of individual initiation events, causing replication to occur approximately at random, failing to produce the required two-fold increase in inhibitor concentration.

Nordstrom et al (1984) have postulated that the low copy number plasmid R1 (3-4 copies per cell), which uses anti-sense RNA to negatively regulate copy number, may replicate according to their "+n" model of control. They have demonstrated that the plasmid encoded initiator protein transcript, repA, is negatively regulated by CopA an

anti-sense RNA; that there is a 1:1 interaction between inhibitor and target and that the rate of repA message synthesis is inversely proportional to the concentration of the constitutively produced, unstable, copA RNA. From this they predict that irrespective of the copy number (i.e. number of origins), on average the same number of replications will occur per cell, such that every cell will receive "+n" copies (where "n" = average copy number prior to cell division) i.e. that the frequency of initiation will be constant in all cells. This model appears to fit the data available for R1 (Gustaffson and Nordstrom 1980; Nielsen and Molin 1984) but cannot explain the progressive decrease in copy number observed with multimerization of pBR322 and pAT153.

The "+n" model predicts that the number of replication events per unit time is directly proportional to the copy number divided by the concentration of repressor.

$$\text{No. of replication events} \propto \frac{\text{Copy Number(origins)}}{[\text{repressor}]}$$

Consider a cell containing 10 plasmid monomers, the origin number is 10 and hence there will be 10 units of repressor. If a cell contains 10 plasmid dimer molecules, then the origin number is 20 and the number of repressor units will also be 20. Consequently, according to this model, the number of replication events for both 1^m and 2^m will be equal and therefore they should have similar copy numbers.

The data of Summers and Sherratt (1984) demonstrated that the copy number of multi-origin rom$^-$ pUC9 plasmids decreased with increasing origin number. A similar result was observed for true multimers of the rom$^-$ pAT153 plasmid and the rom$^+$ pBR322 plasmid. It is probably significant that the copy numbers of pBR322 multimers adhere more closely to the predictions of the "origin-counting" model than the rom$^-$ pAT153 forms. Nevertheless, although Rom may enhance the effect, "origin-counting" appears to occur in situations when RNAI and RNAII are the sole replication controlling elements (Summers and Sherratt 1984).

To explain the origin-counting results it is necessary to hypothesise that plasmid replication occurs in the presence of low concentrations of RNAI. As replication proceeds, the concentration of unstable RNAI increases until a critical level is attained, whereupon binding of RNAI

to RNAII occurs, producing a "stable" RNA-RNA duplex, thereby preventing further replication. A replicating dimer will produce twice the amount of RNAI and Rom protein compared with a plasmid monomer. Consequently, dimer replication will cease when half of the initiation events undergone by a plasmid monomer have occurred.

CHAPTER 4

CAN pSC101*par* STABILIZE ColE1-LIKE PLASMIDS?

Figure 4.1A

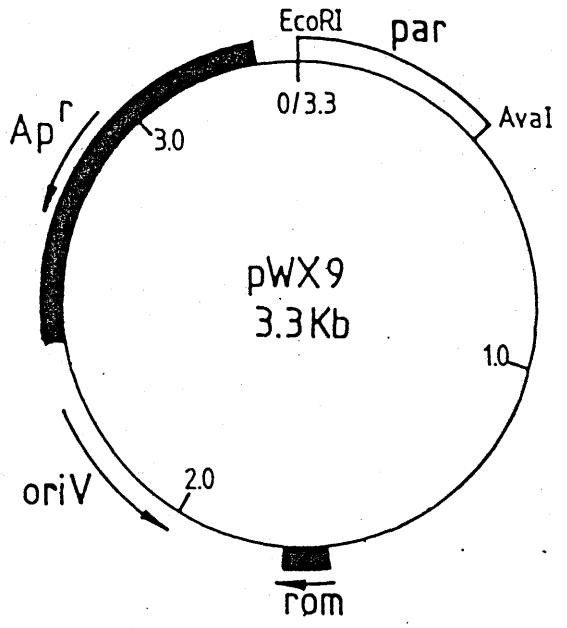


Figure 4.1 B

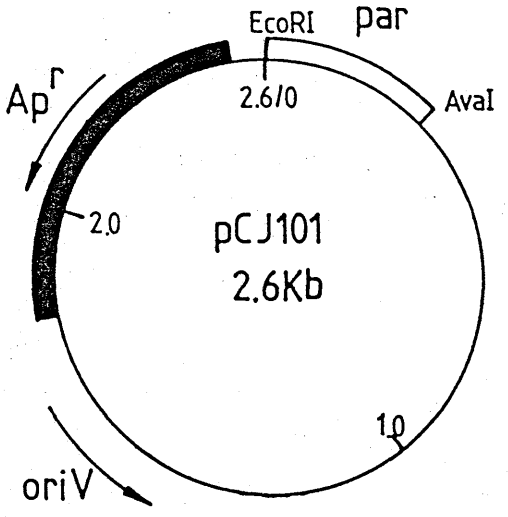


Figure 4.1A + B Plasmid pWX9 and pCJ101. Figure 4.1A is a schematic diagram of pWX9 showing the position of insertion of the par region into the pBR322 replicon. The Tc^r region has been deleted. Figure 4.1B shows plasmid pCJ101 which is pAT153 with par cloned into the same region of the molecule as in pWX9 (pBR322).

INTRODUCTION

A *cis*-acting DNA region of pSC101 designated *par*, was shown to confer stability not only to pSC101 but also to the unrelated plasmid pACYC184 (Meacock and Cohen 1980). This DNA region does not stabilize plasmids in the same manner as *cer*, a site on ColE1 that breaks down plasmid multimers to monomers which therefore can also be considered to be a stability determinant. *par* is thought to cause accurate partitioning of plasmids at cell division by binding to specific sites on the bacterial membrane. Since pAT153 and pBR322 multimers were unstable in DS903 (see Chapter 3) it was of interest to determine whether the *par* region could also stabilize these plasmids.

RESULTS AND DISCUSSION

4.1 Cloning the pSC101 *par* region into pAT153

The plasmid pWX9 (pBR322*par*) was used as a source of the pSC101*par* fragment. This plasmid comprises co-ordinates 1424-4363 of pBR322 (Sutcliffe 1978); the 1424bp EcoR1-Ava1 fragment specifying tetracycline resistance has been replaced by the 372bp EcoR1-Ava1 *par* fragment from pSC101. Consequently, pWX9 is Ap^rTc^s and around 1kb smaller than pBR322 (Fig.4.1A; Derbyshire 1982).

pWX9 DNA was digested with EcoR1 and Ava1 which produced two fragments of 2.9kb and 372bp. The smaller *par* containing fragment was excised from an agarose gel, purified and ligated to the 2.2kb EcoR1-Ava1 fragment from pAT153 to produce the 2.6kb Ap^rTc^s plasmid pCJ101 (Fig.4.1B and 4.2).

4.2 Can *par* Stabilize Multimers of pCJ101 and pWX9?

It was demonstrated in Chapter 3 that only the monomeric forms of both pBR322 and pAT153 were stably maintained in DS903. In order to determine if *par* could stabilize multimeric molecules, pCJ101 and pWX9 DNA was transformed into JC8679, a strain hyper-recombinogenic for plasmids, to facilitate the isolation of multimeric forms.

Specific plasmid multimer bands were excised from agarose gels and the

Figure 4.2

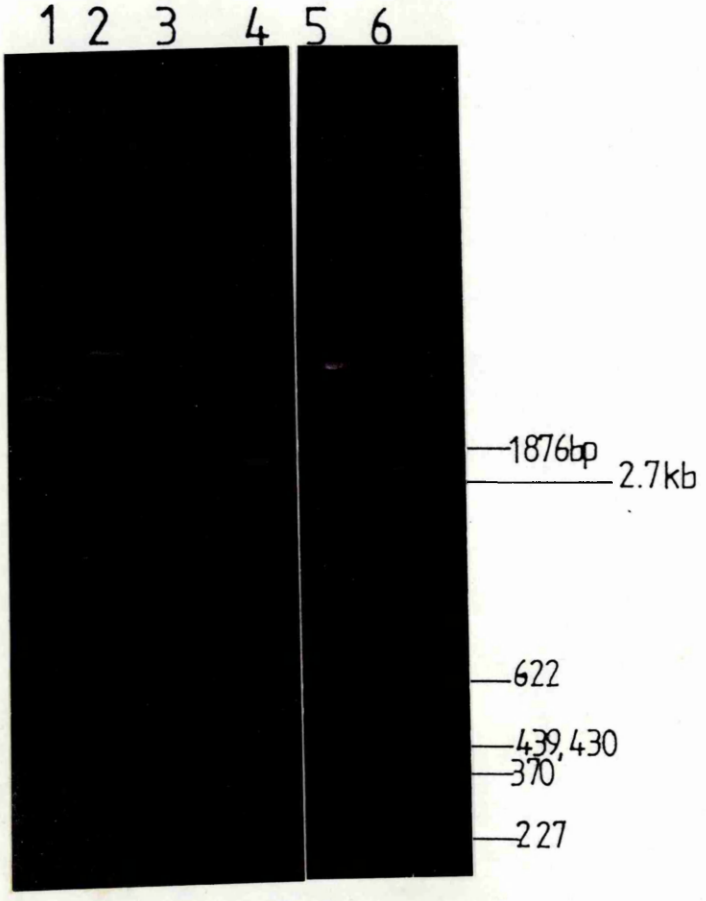


Figure 4.2 Comparative Restriction Digests of pCJ101 and pWX9. 1% agarose gel showing the restriction profiles of PWX9 and pCJ101. Both plasmids possess an EcoRI restriction fragment of approximately 370bp which corresponds to the par fragment.

(1) pCJ101 EcoRI/AvaI
(2) pWX9 EcoRI/AvaI
(3) pBR322 HaeII
(4) pCJ101 s/c
(5) pWX9 s/c
(6) pUC8 2.7kb size marker

Figure 4.3 A

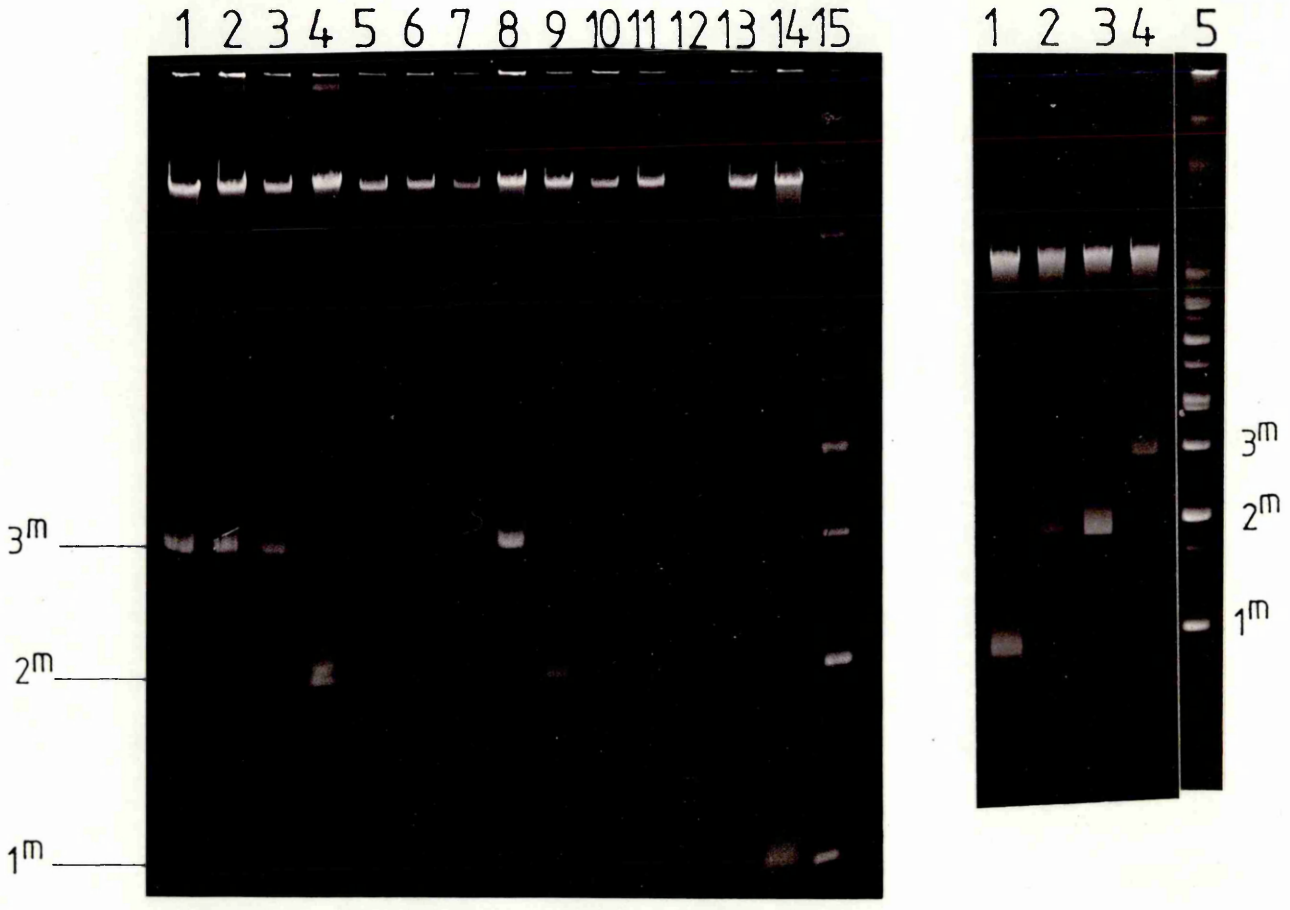


Figure 4.3A + B Multimers of pCJ101 and pWX9. Mutimeric forms of both pWX9 and pCJ101 were isolated from a hyper-recombinogenic strain and used to transform DS903.

4.3A
(1) pCJ101 3^m
(2) " "
(3) " "
(4) pCJ101 2^m
(5) " "
(6) " "
(7) " "
(8) pCJ101 3^m
(9) pCJ101 2^m
(10) " "
(11) " "
(12) -------
(13) pCJ101 2^m
(14) pCJ101 1^m
(15) pCJ101 multimeric DNA

4.3B
(1) pWX9 1^m
(2) pWX9 2^m
(3) " "
(4) pWX9 3^m
(5) pWX9 multimeric DNA

Figure 4.3C D

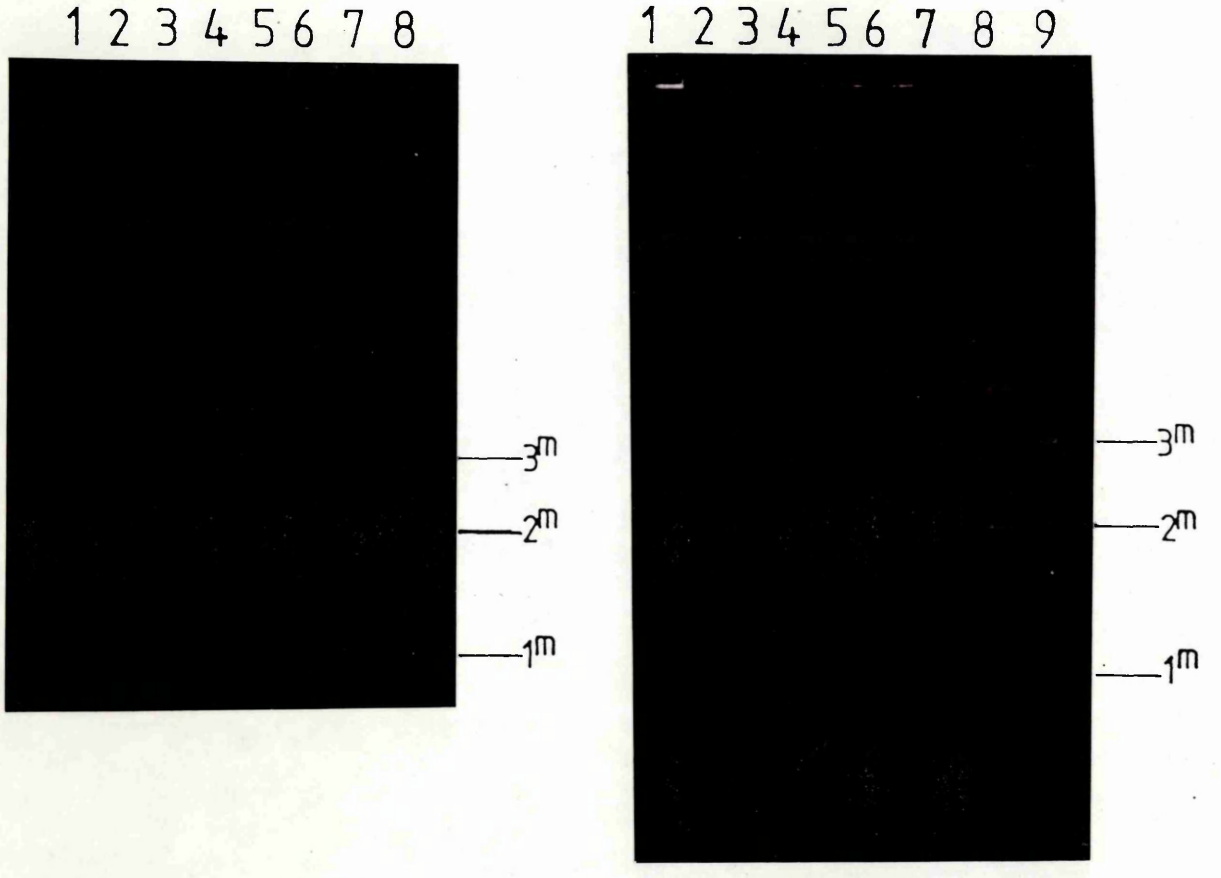


Figure 4.3C + D Higher Multimeric Forms of pCJ101. 0.8% agarose gels showing higher multimers than trimers in track 7 of 4.3C and track 5 of 4.3D.

4.3C
(1) DS903 pCJ101 3^m
(2) " " "
(3) " " "
(4) " " "
(5) " " "
(6) " " "
(7) DS903 pCJ101 $>3^m$
(8) pCJ101 multimeric DNA

4.3D
(1) DS903 pCJ101 1^m
(2) " " "
(3) " " 2^m
(4) " " "
(5) DS903 pCJ101 $>3^m$
(6) " " 3^m
(7) pCJ101 1^m DNA
(8) " 2^m DNA
(9) " 3^m DNA

Figure 4.3E

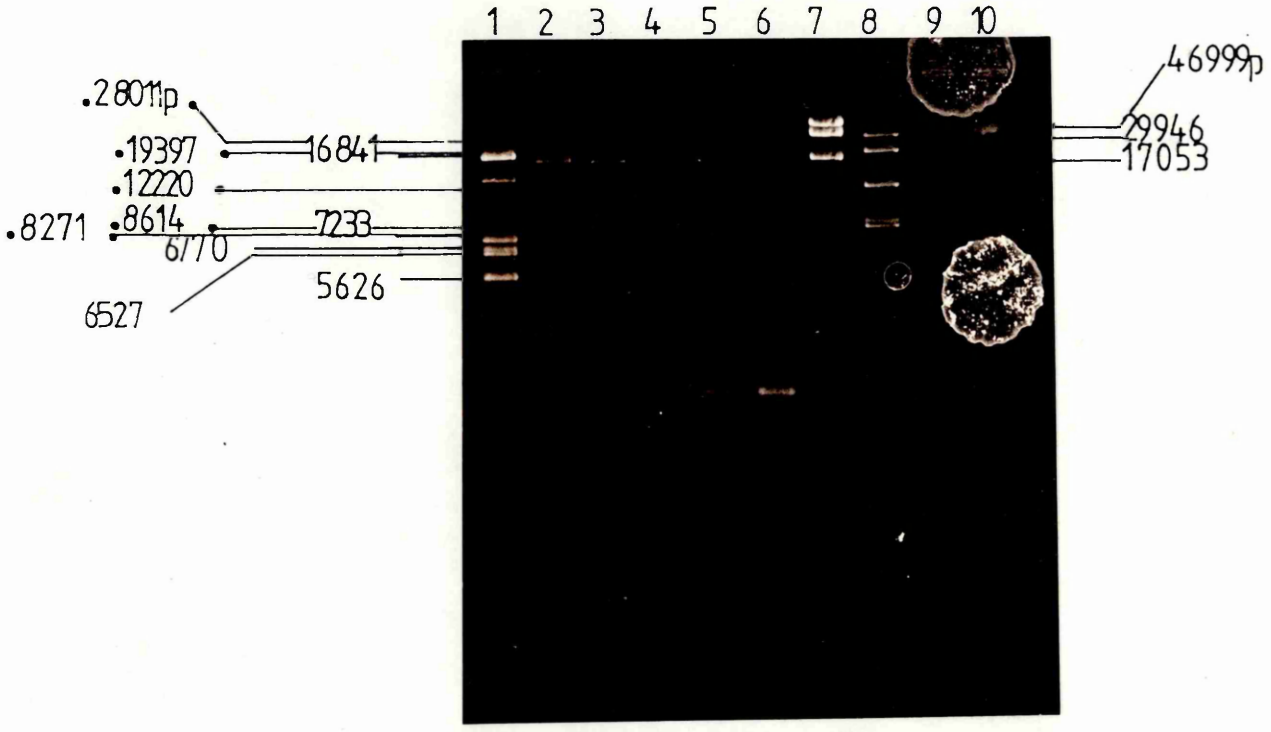


Figure 4.3E Sizing of the Smaller of the Two Higher Multimers of pCJ101. Partial EcoRI digests of the unknown multimeric form of pCJ101 were performed to estimate the size of the oligomeric DNA which was estimated to be approximately 16kb, corresponding to a 6^m.

(1) Lambda BamHI
(2) pCJ101 unknown EcoRI 2'
(3) " " " 4'
(4) " " " 6'
(5) " " " 8'
(6) " " " 10'
(7) Lambda KpnI
(8) Lambda SmaI
(9) supercoil of pCJ101 multimeric form
(10) " " " " "

isolated DNA used to transform DS903. Subsequent single colony gel analysis demonstrated that dimeric and trimeric plasmid containing strains had been constructed (Fig.4.3A and B). Higher oligomeric forms of pCJ101 were also isolated as shown in figure 4.3C track 7 and 4.3D track 5. An attempt to determine the size of the smaller of these two oligomers is shown in figure 4.3E. Sizing involved partial restriction at the unique EcoRI site over a short time course. After 2 minutes considerable restriction had occurred, however, by comparing the position of the bands in the 2 minute track to the supercoiled DNA in tracks 9 and 10, the linear form was estimated to be approximately 16kb, which corresponds to a pCJ101 6^m. The larger oligomer shown in figure 4.3D was not characterized, but by reference against a pCJ101 multimer "ladder" was estimated to be at least an 8^m.

The stability of the various plasmid forms was investigated by batch growth in non-selective medium for 100 generations. Figure 4.4 shows the comparative stabilities of pAT153, pCJ101 and pWX9 1^m,2^m and 3^m and pCJ101 6^m and 8^m and demonstrates that the *par* region stabilizes 2^m and 3^m forms of both pCJ101 and pWX9 but not the 6^m and the possible 8^m of pCJ101.

The ability of these plasmids to multimerize in JC8679 demonstrated that *par* does not act in a similar manner to ColE1*cer*, P1*lox*P or CloDF13*par*B which are sites at which site-specific recombination occurs to resolve plasmid multimers to monomers (Summers and Sherratt 1984; Hoess *et al* 1984; Hakkaart *et al* 1984). Similarly, the persistence of multimers in DS903 is also good evidence that *par* is dissimilar to *cer*.

4.3 The pSC101 *par* Region Cannot Stabilize all Plasmid Vectors.

Another *par*$^+$ consruct was available for study. The plasmid pST2 (obtained from G.D. Searle) comprises a complete pAT153 replicon with the pSC101 *par* region cloned via EcoR1 linkers into its EcoR1 site (W.Tacon pers.comm. Fig.4.5A and B). The Ava1 digest of pST2 shown in figure 4.5B demonstrates the orientation of the *par* fragment. The stability of the monomeric and multimeric forms of this plasmid were investigated. pST2 DNA was transformed into JC8679, specific multimeric forms purified from agarose gels and the DNA used to transform DS903. The isolation of dimeric and trimeric forms of the plasmid was confirmed by single colony gel analysis (Fig.4.6).

Figure 4.4

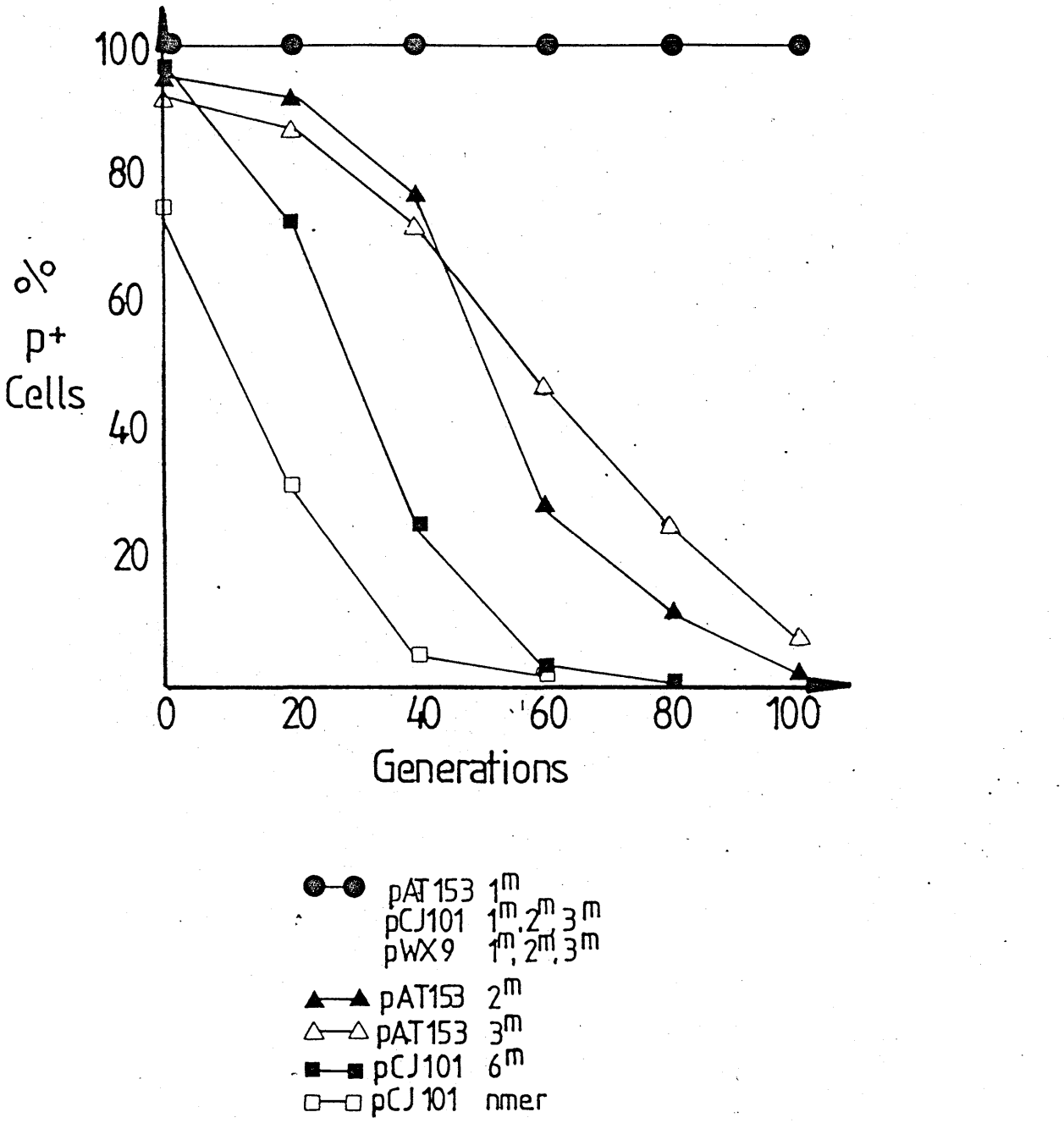


Figure 4.4 Stability Comparison Between pAT153, pCJ101 and pWX9 Multimers. The stability graph demonstrates that pCJ101 and pWX9 1^m, 2^m and 3^m are stably maintained for 100 generations under non-selective growth however higher forms ($>3^m$) of pCJ101 are not. The stability graphs of 2^m and 3^m of the parent plasmid pAT153 are included for comparison.

Figure 4.5A

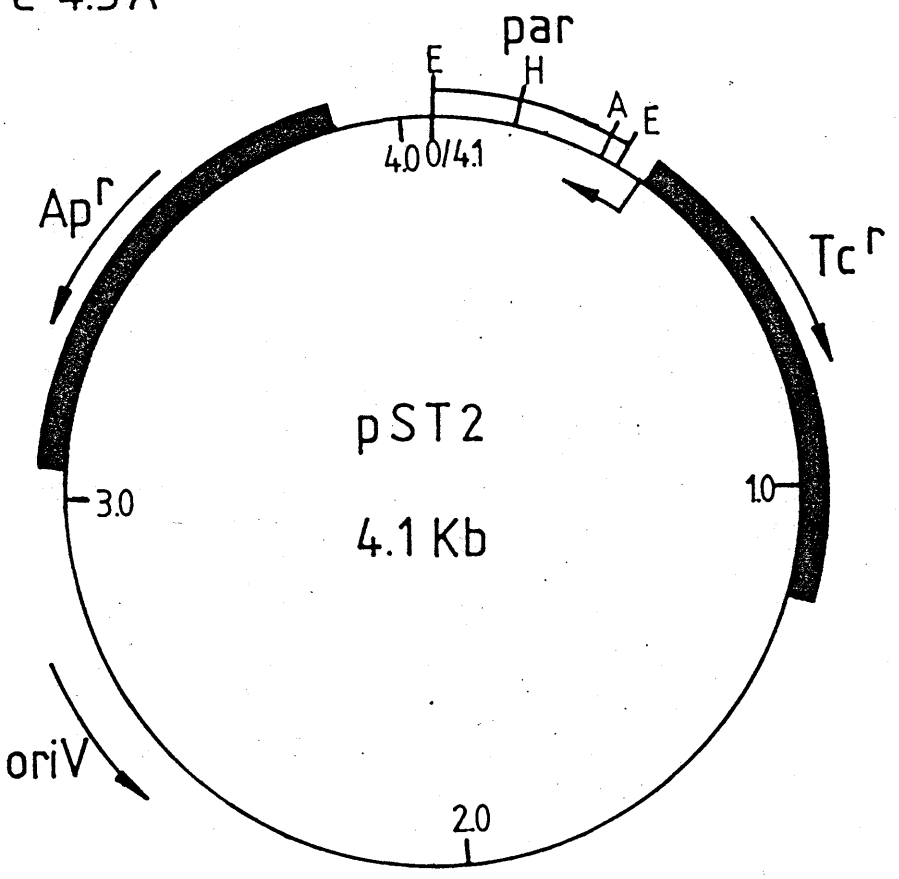


Figure 4.5A Schematic Diagram of pST2. Plasmid pST2 carries the pSC101 par fragment cloned at the unique EcoRI site via EcoRI linkers. Unlike pCJ101 this plasmid possesses an intact Tc^r region. The orientation, of the par fragment (determined in figure 4.5B). is shown by the asymmetrically located HaeII site within the cloned fragment

Subsequent stability experiments demonstrated that the par region failed to stabilize multimeric forms of pST2: the 2^m and 3^m forms had segregation frequencies of 1.2×10^{-2} and 1.8×10^{-2} per cell per generation respectively (mean over 100 generations). (Fig.4.7). These frequencies were very similar to those observed for pAT153 2^m and 3^m (Chapter 3 section 3.7) and demonstrated that par did not enhance the stability of these plasmids. The monomeric form of pST2 like pAT153, was fully stable.

4.4 Does par Stabilize pCJ101 Multimers by Affecting Copy Number?

The apparent inability of par to stabilize pST2 multimers could be due to an intrinsic feature of this plasmid construct. It is possible that the stabilization observed for pCJ101 2^m and 3^m is due to an increase in copy number and not as a direct result of active partitioning. To try to determine which of these two possibilities was correct, plasmid copy number estimations were carried out using the in vivo labelling technique discussed in chapter 3. Table 4.1 displays the calculated copy numbers for pCJ101 and pST2 multimers. These results demonstrate that the copy numbers of the 2^m and 3^m species of each plasmid do not differ greatly. It is likely therefore, that par is stabilizing pCJ101 multimers but not pST2 multimers.

Initially, the relative copy numbers of pAT153 1^m and pCJ101 1^m were assessed by single cell resistance to cephaloridine. Using this method, the copy number of pCJ101 1^m appeared to be much lower than that of pAT153 1^m, since the plasmid containing strains had LD_{50} values of 9 and 23 ug/ml respectively (Fig.4.8). The data from the in vivo labelling experiments dispute this result however, since pCJ101 1^m and pAT153 1^m have copy numbers of 29 and 23 per genome equivalent respectively (copy number of pAT153 1^m was given in chapter 3 Table 3.2).

This discrepancy can be explained by the deletion of the tetracycline resistance region during the construction of pCJ101. The presence of the strong "anti-tet" promoter has been shown to contribute to the expresion of the bla gene in pBR322 (Stueber and Bujard 1981; Von Gabain et al 1983) and probably accounts for the differing levels of cephaloridine resistance of pCJ101 and pAT153 since pAT153, unlike pCJ101, possesses the "anti-tet" region.

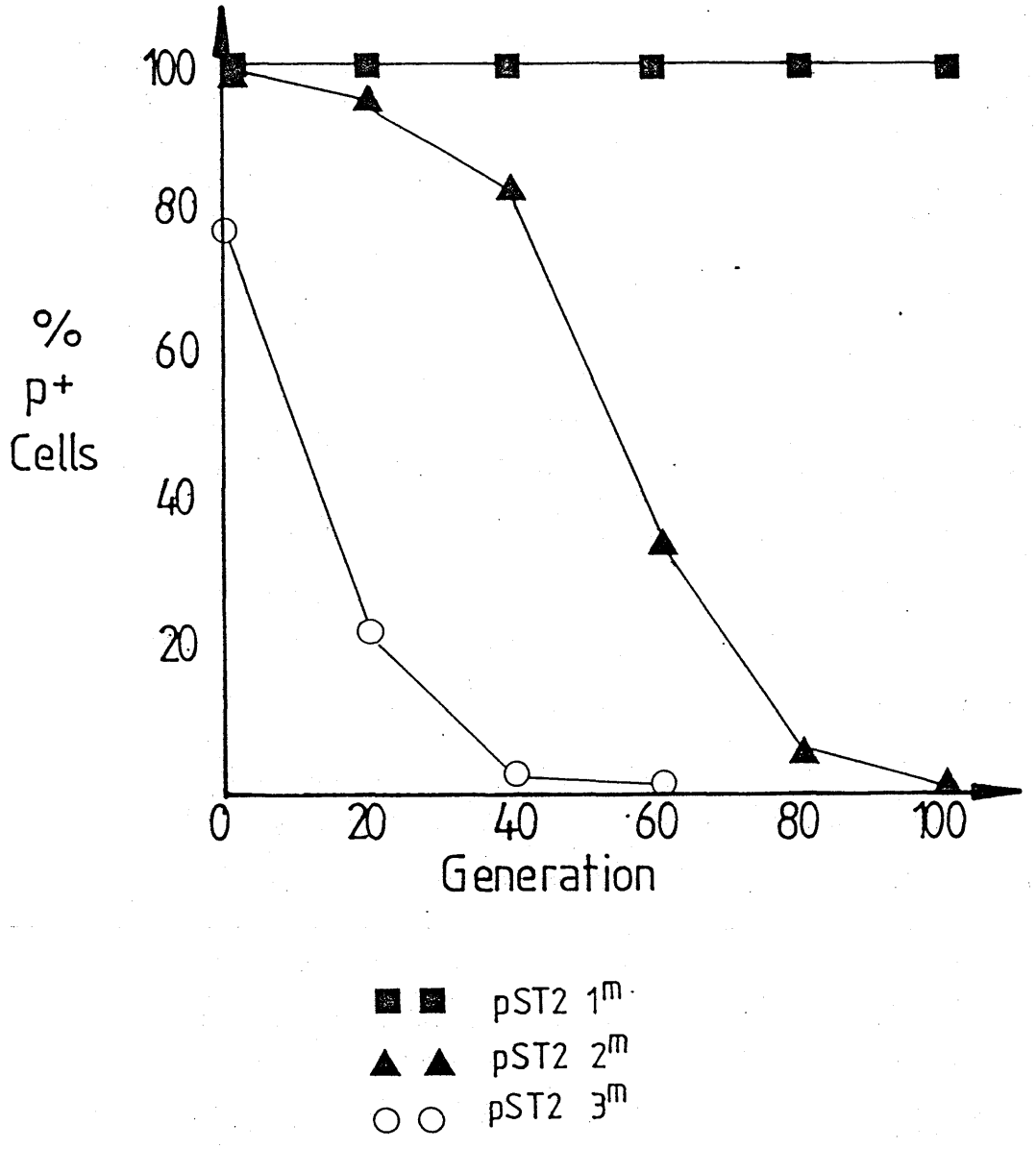


Figure 4.7 Stability of pST2 Multimers in DS903. The segregation frequencies of each plasmid are shown below.

DS903 pST2 1^m $<10^{-5}$ per cell per generation
DS903 pST2 2^m 1.2×10^{-2} mean value per cell per generation (100 gen.)
DS903 pST2 3^m 1.8×10^{-2} " " " " " " (60 gen.)

Strain	Plasmid	Plasmid Size (kb)	Calculated Copy Number			% Standard Deviation
			(a)	(b)	(c)	
DS903	pCJ101 1^m	2.62	29	+/-4.3	3	15%
DS903	pCJ101 2^m	5.24	17	+/-2.9	3	17%
DS903	pCJ101 3^m	7.86	14	+/-2.8	3	20%
DS903	pST2 1^m	4.0	26	+/-4.7	3	18%
DS903	pST2 2^m	8.0	16	+/-2.4	3	15%
DS903	pST2 3^m	12.0	12	+/-2.3	3	19%

Table 4.1 Calculated Copy Number of pCJ101 and pST2 from *in vivo* Labelling Data. Plasmid copy numbers were calculated per genome equivalent based on a figure of 3.8×10^3kb for the size of a non-replicating *E.coli* chromosome. The calculated copy numbers per genome equivalent are given in column (a), the standard deviations in column (b) and the number of independent trials in column (c). The calculation used is given below:

$$\text{Copy Number} = \frac{\dfrac{\text{cpm plasmid}}{\text{cpm chromosome}} \times \text{Size } \textit{E.coli} \text{ Chromosome (kb)}}{\text{Size of Plasmid (kb)}}$$

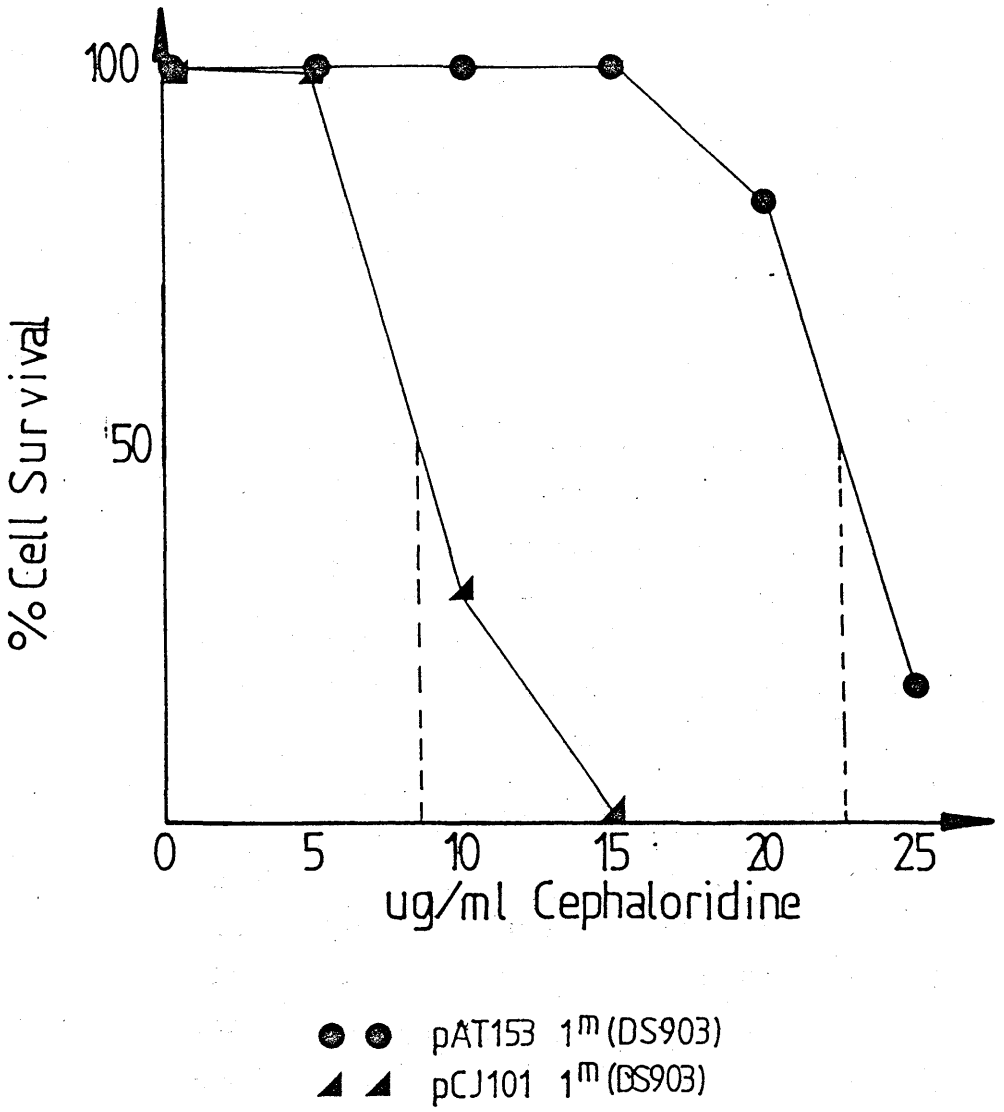


Figure 4.8 Comparison of the Cephaloridine Resistance Levels of Exponentially Growing pCJ101 and pAT153 Containing Cells.

4.5 Why Does *par* Function in pCJ101 and pWX9 but not in pST2?

It seems paradoxical that *par* can stabilize pCJ101 and pWX9 multimers but not the similar pST2 plasmid. The only difference between pCJ101 and pST2 is the presence of the tetracycline resistance region in the latter (Fig.4.1B and 4.5A) and it is possible that the transcriptional environment around *par* can affect its function.

Meacock and Cohen (1980) first demonstrated that pSC101*par* could fully stabilize pACYC184, an unrelated, unstable, multicopy plasmid. From this and other data they concluded that the partition locus was effective, irrespective of the location and orientation on a plasmid and its action was independent of the replicon type. Recent research however, has shown that whilst *par* can improve the stability of some vectors (pBR322 and pACYC184 carrying the tryptophan operon of *E.coli*), it cannot fully stabilize them. Transcriptional readthrough into *par* was considered to be a likely explanation for the reduced ability of *par* to function (Skogman *et al* 1983). These data imply that the location of *par* within a plasmid may be very important for *par* function.

Sequence analysis of the partition locus of pSC101 revealed a putative Rho-independent transcription terminator situated between co-ordinates 170-206 (Miller *et al* 1983; Fig.4.9). Using a transcription terminator probe vector pUB10, the terminator was found to function in only one orientation (Tucker *et al* 1984; Fig.4.9). This terminator may not be crucial to *par* function (see Concluding Remarks).

4.6 Does Transcriptional Readthrough into *par* Affect its Ability to Function?

In an attempt to determine if transcription through the fragment disrupted *par* function, a 411bp HaeIII *par* containing fragment from pCJ101 was cloned into the pUC8 HincII site in the polylinker. Figure 4.10A demonstrates the presence of the EcoRI-AvaI *par* fragment in pUC8*par* (pCJ122). The orientation of this fragment was determined by a HaeII digest since there is an asymmetrically placed HaeII site within *par* (Fig.4.9). The restriction profile obtained (Fig.4.10B) was compatible with the orientation shown in figure 4.10C. In this orientation, transcription originating from the strong P_{lac} promoter traverses the *par* region in the same direction as transcription from the

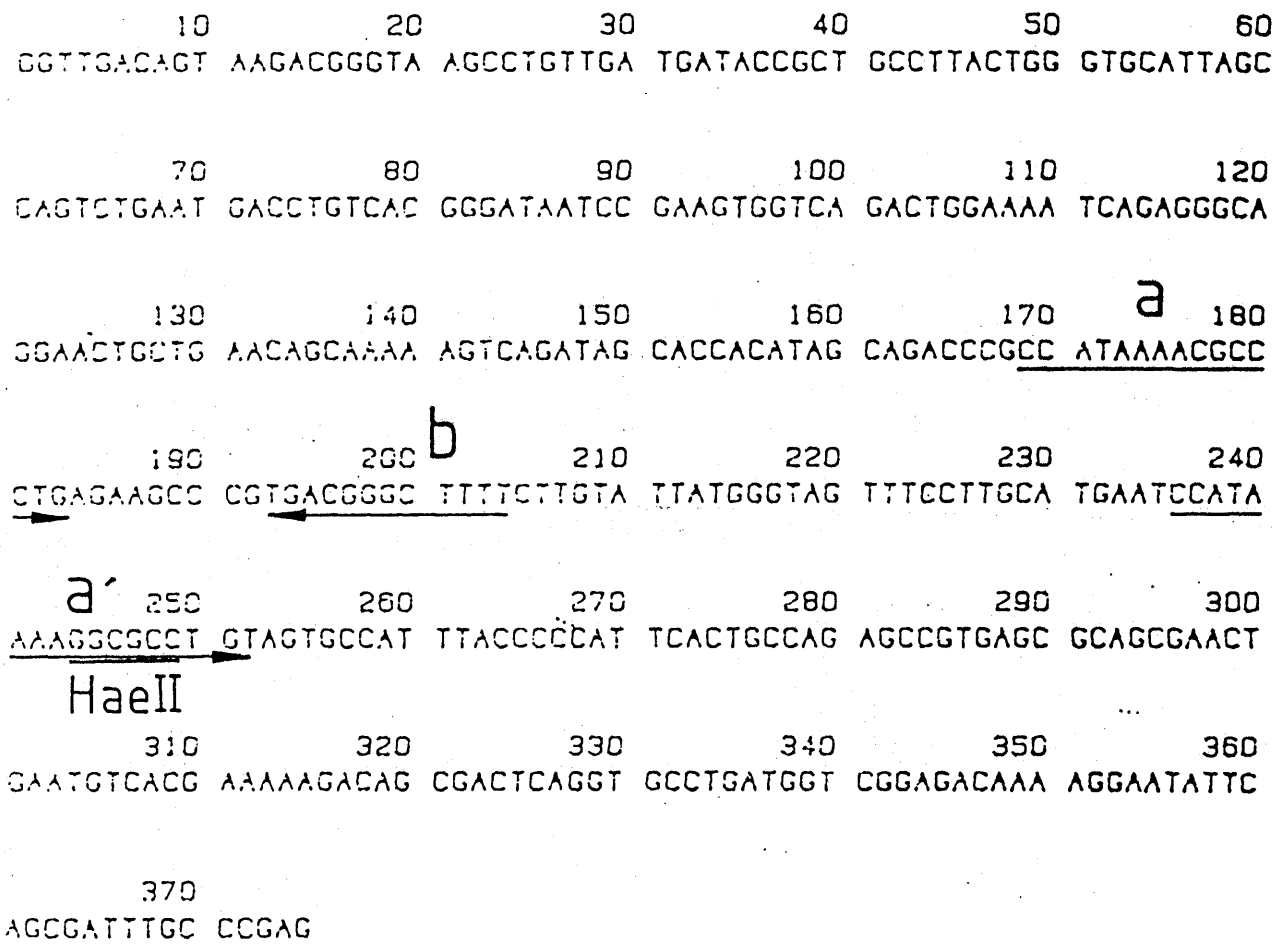


Figure 4.9 The pSC101 par Fragment. Highlighted in the sequence above are the three "partition related segments" a, b and a'. The a, b repeat region is a terminator-like structure believed to be active in vivo. The asymmetrically located HaeII site is also shown.

Figure 4.10A

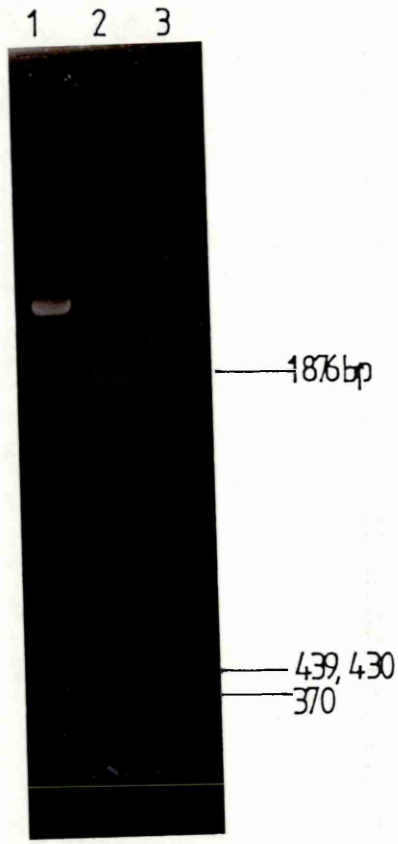


Figure 4.10A Restriction Analysis of pCJ122 and pCJ101. 5% acrylamide gel demonstrating the presence of the 372bp par containing EcoRI/AvaI fragment in both pCJ122 and pCJ101.

(1) pCJ122 EcoRI/AvaI
(2) pAT153 HaeII
(3) pCJ101 EcoRI/AvaI

Figure 4.10B

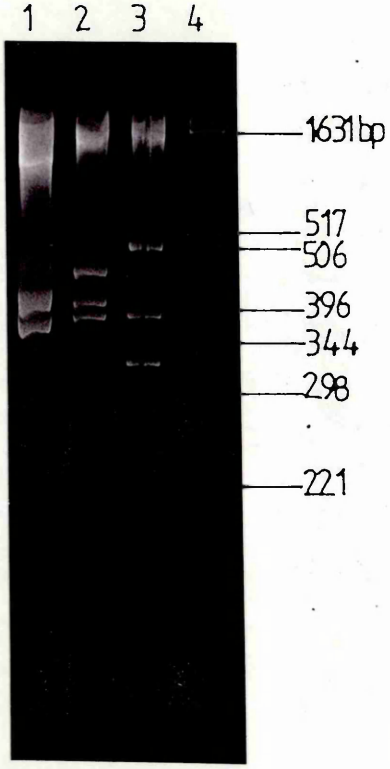


Figure 4.10B Restriction Analysis of pCJ122 and pCJ123. 1% agarose gel showing HaeII restriction pattern of pCJ122 and pCJ123. The restriction patterns show that the orientation of par is reversed in pCJ123 with respect to pCJ122.

(1) pUC8 HaeII
(2) pCJ122 HaeII
(3) pCJ123 HaeII
(4) pBR322 HinFI

"anti-tet" promoter in pST2 (Fig.4.5A).

To obtain a clone containing the par fragment in the opposite orientation, the par containing polylinker from pCJ122 was removed using EcoRI-HindIII and then ligated to pUC9 EcoRI-HindIII restricted DNA. Since in pUC9 the polylinker is reversed with respect to pUC8, the par fragment also becomes reversed upon cloning with these enzymes (Fig.4.10B track 3; 4.10D). In this orientation, the transcription terminator within par (Fig.4.9) should decrease the level of transcription traversing the important regions of the fragment (Tucker et al 1984).

If transcriptional readthrough affects the efficacy of par, one predicts that a difference in the stabilities of pUC8par (pCJ122) and pUC9 par (pCJ123) should be observed. To test this, the stabilities of pUC8, pUC9, pCJ122 and pCJ123 monomers in DS903 were determined (Fig.4.11). The stability data show that both pCJ122 and pCJ123 1^m plasmids are stabilized by the presence of par implying that transcriptional readthrough from P_{lac} does not disable par function in these constructs. This would imply that the instability observed in pST2 may not be due to readthrough transcription from the "anti-tet" promoter alone. However, pST2 instability was only detected in 2^m and 3^m and so a direct comparison between 1^m pCJ122 and pCJ123 plasmids may not be valid.

4.7 Is par Unable to Stabilize Plasmid Multimers?

Multimeric forms of pST2, unlike the monomer are unstable in DS903. Since the parent plasmid pAT153 1^m is stable, it was unknown whether par functioned at all in pST2, or if multimeric plasmids could not be stabilized by the par region. Since both pCJ122 and pCJ123 1^m plasmids were both fully stabilized by par, it was possible using these plasmids to determine the effect of par on plasmid multimers. pCJ122 and pCJ123 2^m and 3^m plasmids were isolated, transformed into DS903 (Fig.4.12A and B) and their stability over 100 generations analysed. Figure 4.13 shows the stability of pUC8, pUC9, pCJ122 and pCJ123 multimers. Dimeric and trimeric pCJ123 plasmids are unstably maintained, having segregation frequencies similar to their par$^-$ counterparts. pCJ122 dimer is stably maintained in contrast to the trimeric plasmid which has a segregation frequency identical to pUC8 3^m. Copy number determination experiments

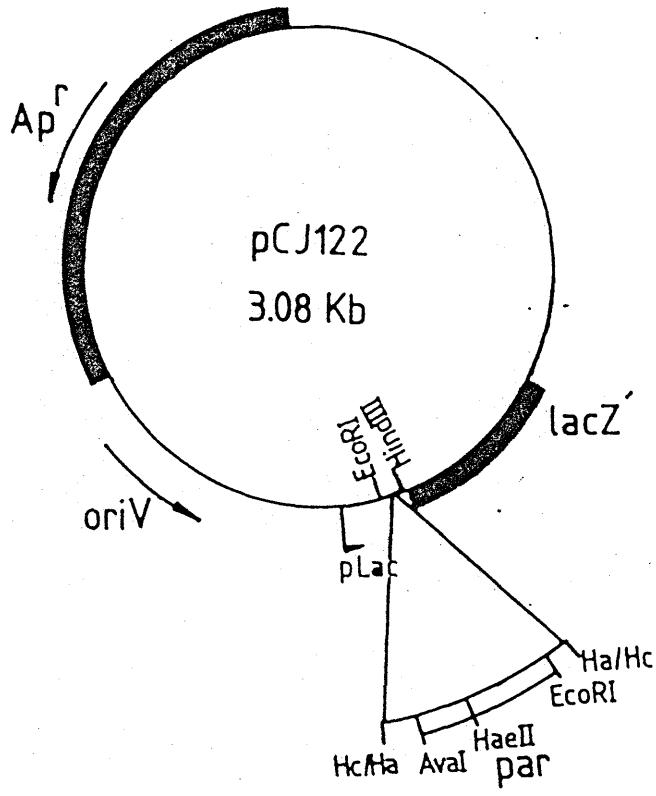


Figure 4.10 D

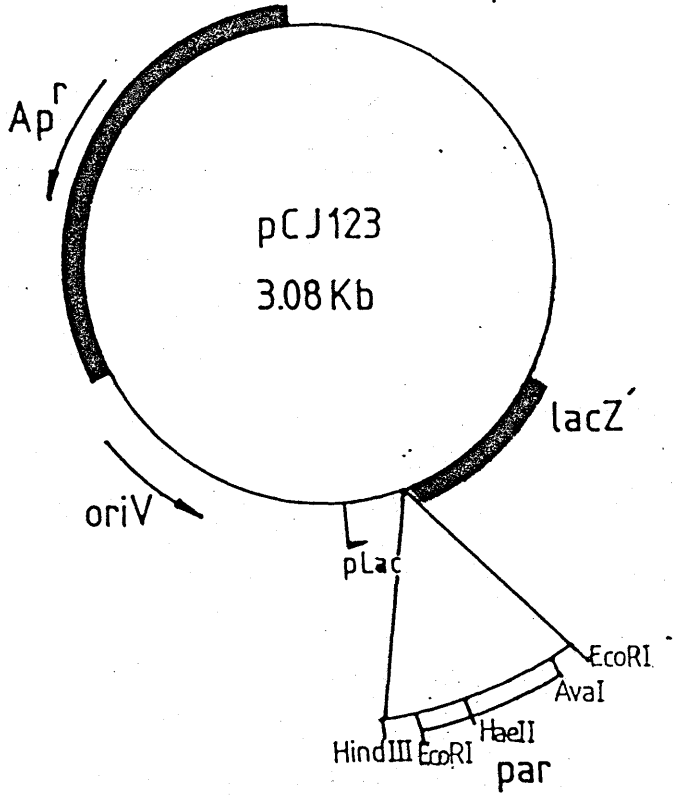


Figure 4.10C + D Schematic Diagrams of pCJ122 and pCJ123 Showing the Orientation of the cloned par fragment in each Plasmid.

Figure 4.11

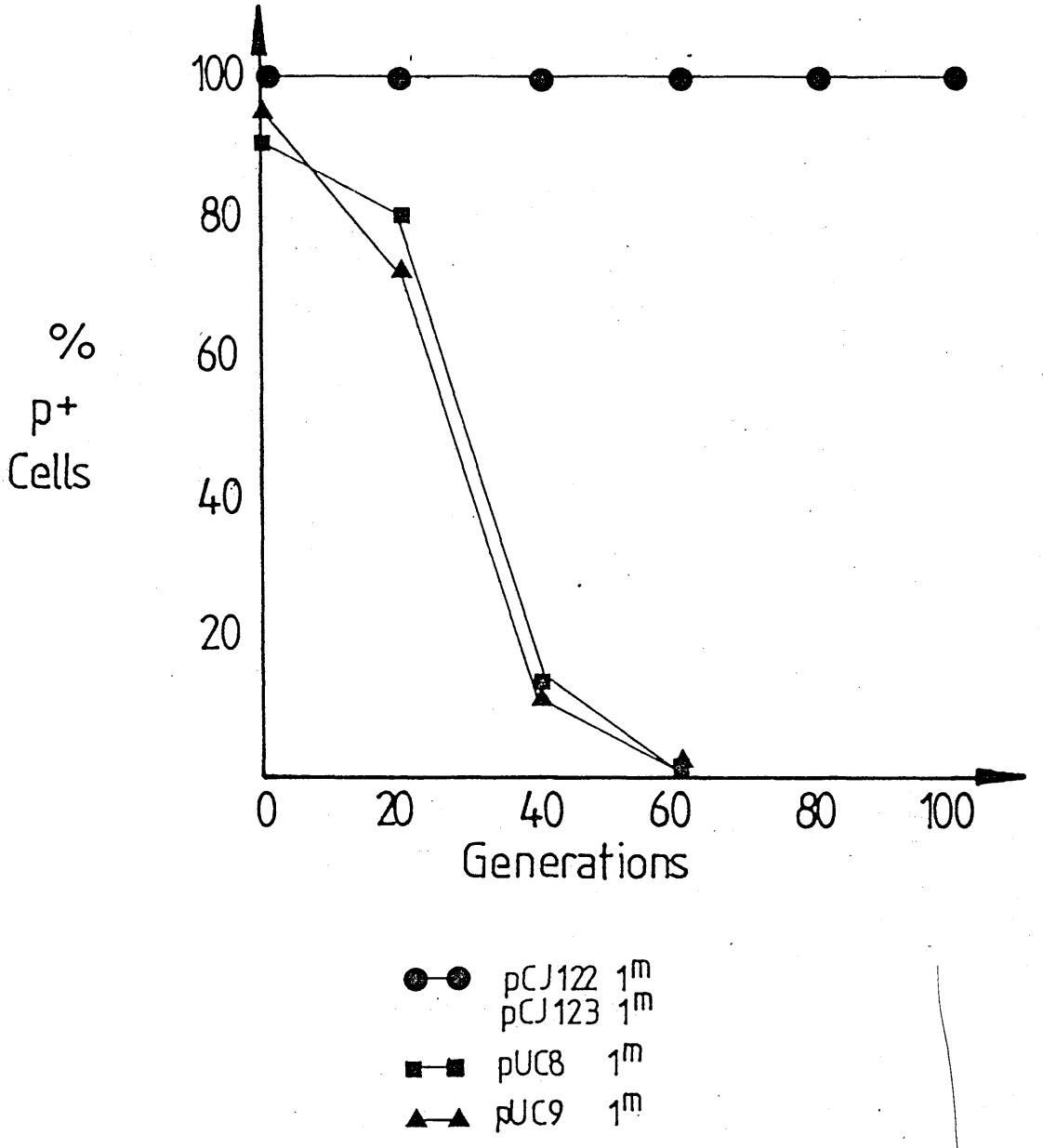


Figure 4.11 Stability Comparison of pUC8+9 with pCJ122 and pCJ123. The presence of the par fragment stabilizes pUC8 and pUC9 1^m.

Figure 4.12A

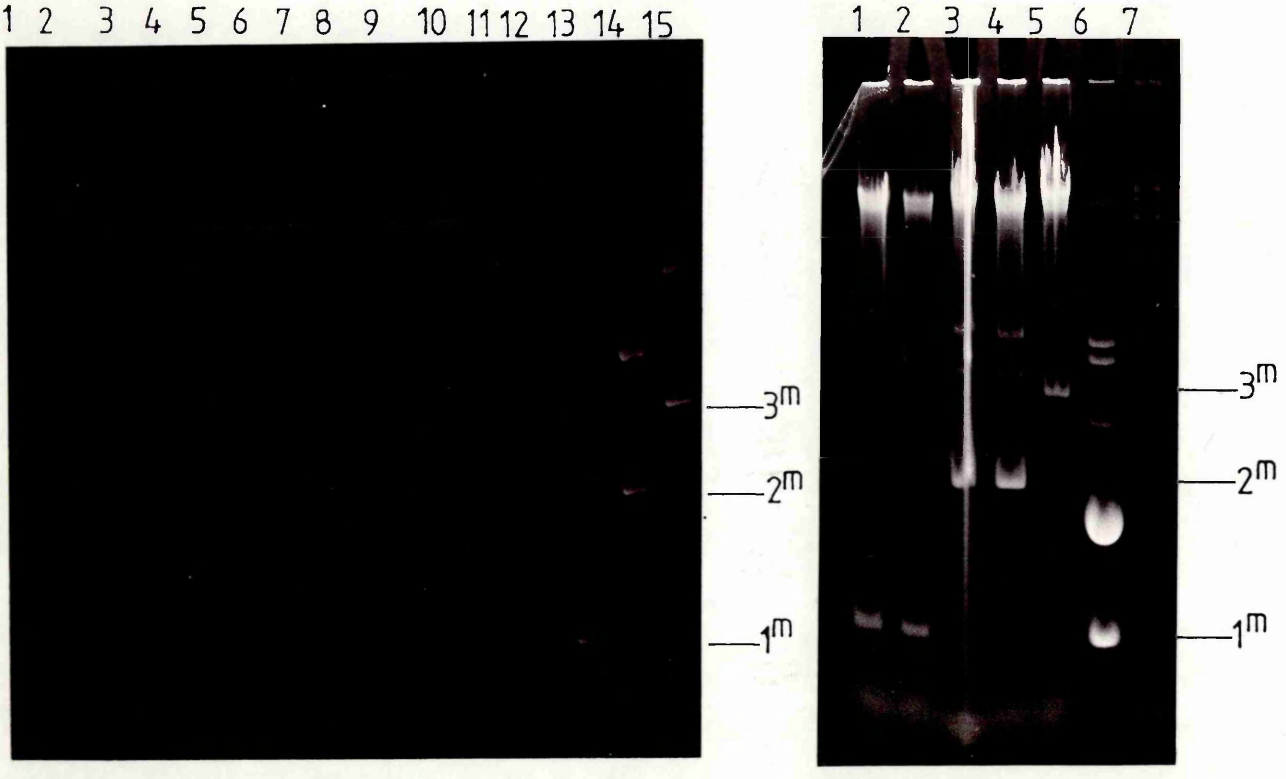


Figure 4.12A + B Multimers of pCJ122 and pCJ123. 0.8% agarose single colony gels showing 1^m, 2^m and 3^m of pCJ122 and pCJ123.

4.12A
(1) DS903 pCJ122 1^m
(2) " " "
(3) " " "
(4) " " "
(5) DS903 pCJ122 2^m
(6) " " "
(7) " " "
(8) " " "
(9) DS903 pCJ122 3^m
(10) " " "
(11) " " "
(12) " " "
(13) pCJ122 1^m DNA marker
(14) " 2^m " "
(15) " 3^m " "

4.12B
(1) DS903 pCJ123 1^m
(2) " " "
(3) DS903 pCJ123 2^m
(4) " " "
(5) DS903 pCJ123 3^m
(6) pCJ123 1^m DNA
(7) multimeric pCJ123 DNA

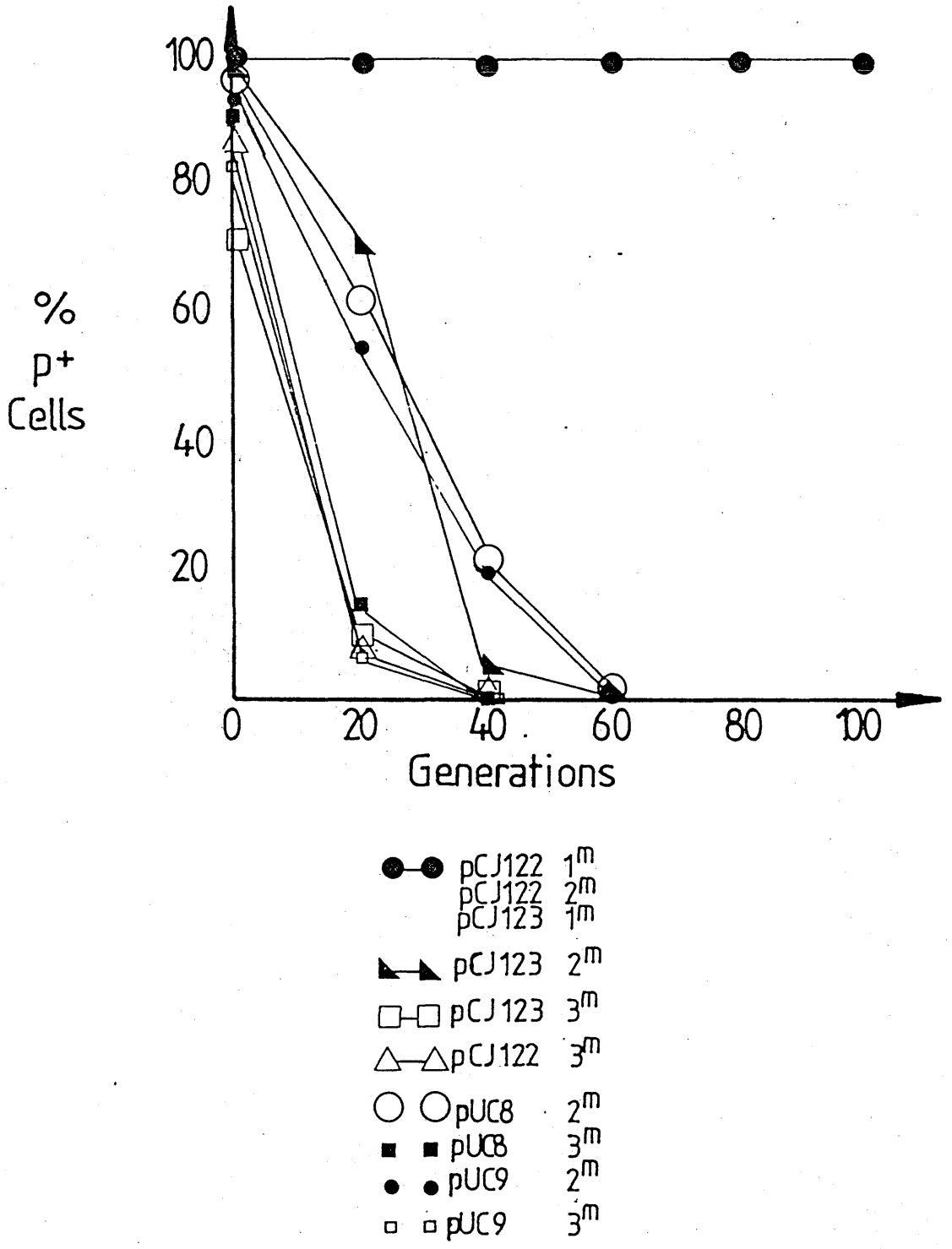


Figure 4.13 Stability Comparison of Multimeric Forms of pCJ122, pCJ123 pUC8 and pUC9 in DS903. The segregation frequencies for the unstable plasmids are given below.

pCJ101	3^m	4×10^{-2}	per cell per generation
pCJ123	2^m	2×10^{-2}	" " " "
pCJ123	3^m	3.1×10^{-2}	" " " "
pUC8	2^m	1.6×10^{-2}	" " " "
pUC8	3^m	3.7×10^{-2}	" " " "
pUC9	2^m	1.9×10^{-2}	" " " "
pUC9	3^m	3.7×10^{-2}	" " " "

demonstrated that the copy numbers of pCJ122 1^m, 2^m, 3^m and pCJ123 1^m, 2^m, 3^m plasmids were similar to their pUC8 and pUC9 counterparts (see Table 4.2).

These data imply that multimer instability does not result from a reduction in copy number due to the presence of *par*. This allied to the segregation frequencies of *par*$^+$/*par*$^-$ multimers suggests that *par* does not function in most multimeric, multicopy plasmids. The stabilization of pCJ122 2^m indicates that there may be a preferential orientation for *par* function which becomes apparent in multimers.

4.8 Do Multiple *par* Regions on a Plasmid Molecule Prevent *par* Function?

The observation that *par* cannot stabilize all multimeric molecules led to the suggestion that perhaps the physical presence of multiple *par* regions on some plasmids abolishes *par* activity.

Deletion analysis has demonstrated that the "functional" region of *par* lies within co-ordinates 68-258 (Tucker *et al* 1984; Kalla and Gustafsson 1984; Fig 4.9). This region includes the three repeat sequences designated a, b and a'. Computer gene search analyses failed to detect the initiation of likely open reading frames in the region 4500-5450 of pSC101 (Bernardi and Bernardi 1984) although *par* lies entirely within this region.

The *cis*-acting nature of *par* supports the non-coding argument and it has been suggested that *par* may function as a site which binds the plasmid to some cellular apparatus which ensures faithful segregation. A good candidate for this role is the bacterial cellular membrane and indeed a specific binding between the outer membrane and the pSC101*par* region has been demonstrated by Gustafsson *et al* (1983). If there are a limited number of cellular sites at which binding can occur, it is possible that these become "clogged" with multiple *par* regions on one molecule and prevent active partitioning. On the other hand, *par* may be unable to function on a molecule possessing more than one replication origin.

4.8.1 CONSTRUCTION OF MULTIPLE REPLICATION ORIGIN PLASMIDS CONTAINING ONE *PAR* REGION.

In an attempt to distinguish between these two possibilities, pUC9

Strain	Plasmid	Size (kb)	Calculated Copy Number			% Standard Deviation
			(a)	(b)	(c)	
DS903	pUC8 1^m	2.7	118	+/-23.6	3	20%
DS903	pUC8 2^m	5.4	88	+/-15.84	3	18%
DS903	pUC8 3^m	8.1	64	+/-13.4	3	21%
DS903	pCJ122 1^m	3.0	125	+/-21.25	3	17%
DS903	pCJ122 2^m	6.0	93	+/-16.7	3	18%
DS903	pCJ122 3^m	9.0	70	+/-15.4	3	22%
DS903	pUC9 1^m	2.7	123	+/-23.37	3	19%
DS903	pUC9 2^m	5.4	81	+/-13.7	3	17%
DS903	pUC9 3^m	8.1	68	+/-10.8	3	16%
DS903	pCJ123 1^m	3.0	115	+/-23.0	3	20%
DS903	pCJ123 2^m	6.0	91	+/-16.38	3	18%
DS903	pCJ123 3^m	9.0	78	+/-17.9	3	23%

Table 4.2 Calculated Copy Numbers from *in vivo* Labelling Data. Plasmid copy numbers were calculated per genome equivalent based on a figure of 3.8×10^3kb for the size of a non-replicating *E.coli* chromosome. The calculated copy numbers per genome equivalent are given in column (a), the standard deviations in column (b) and the number of independent trials in column (c). The copy number calculation was as follows:

$$\text{Copy Number} = \frac{\dfrac{\text{cpm plasmid}}{\text{cpm chromosome}} \times \text{Size } \textit{E.coli} \text{ Chromosome (kb)}}{\text{Size of Plasmid (kb)}}$$

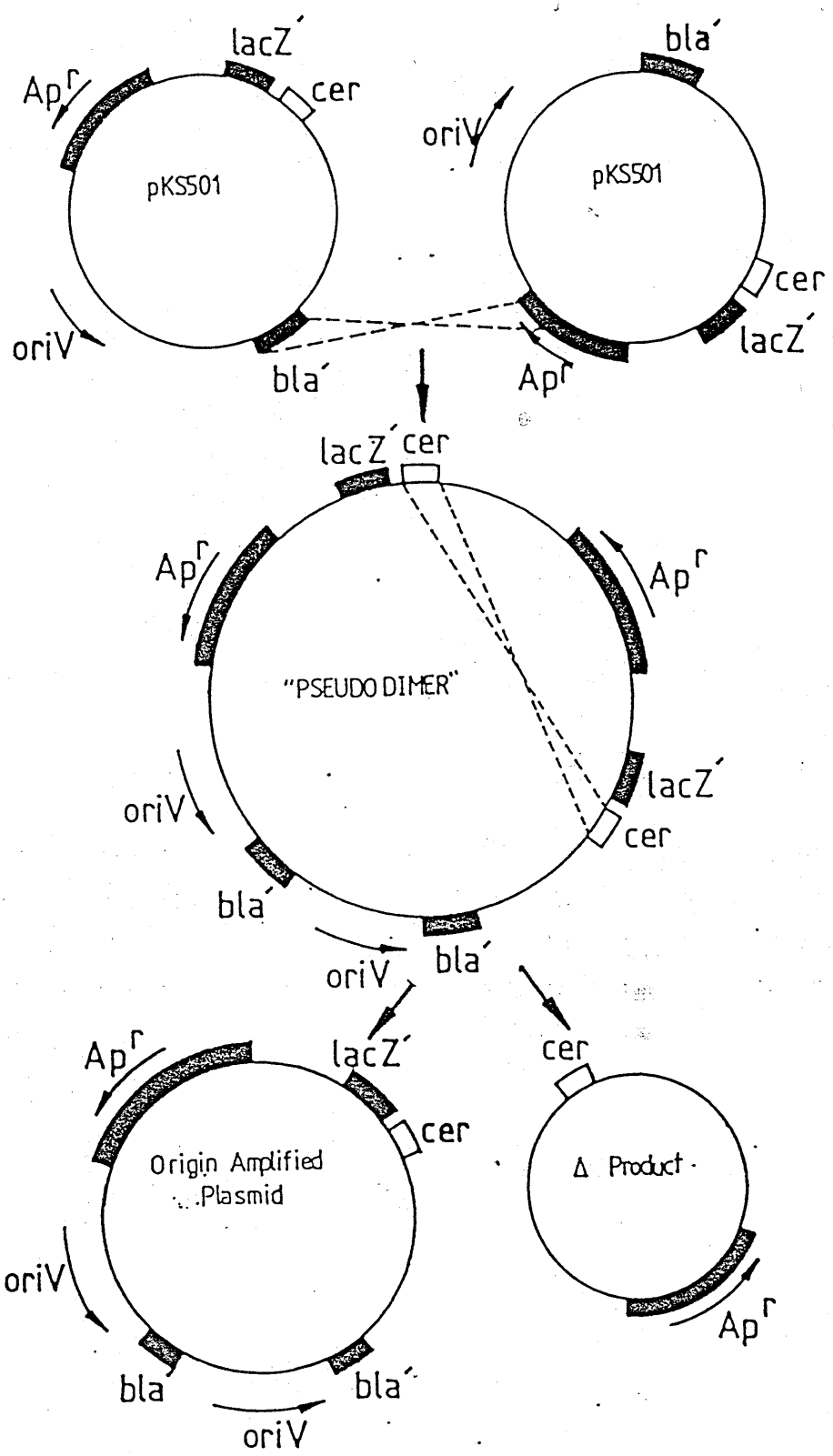


Figure 4.14 Schematic Diagram of the "Origin-Amplifying" Vector pKS501. Intermolecular unequal crossing-over between the 450bp bla' region and the end of the intact bla gene results in the formation of a pseudo-dimer. Intramolecular recombination between cer sites resolves the molecule into a plasmid which contains 2 origins and a deletion product which does not replicate because it lacks a replication origin. Consequently, it is not detected on gels.

Figure 4.15A

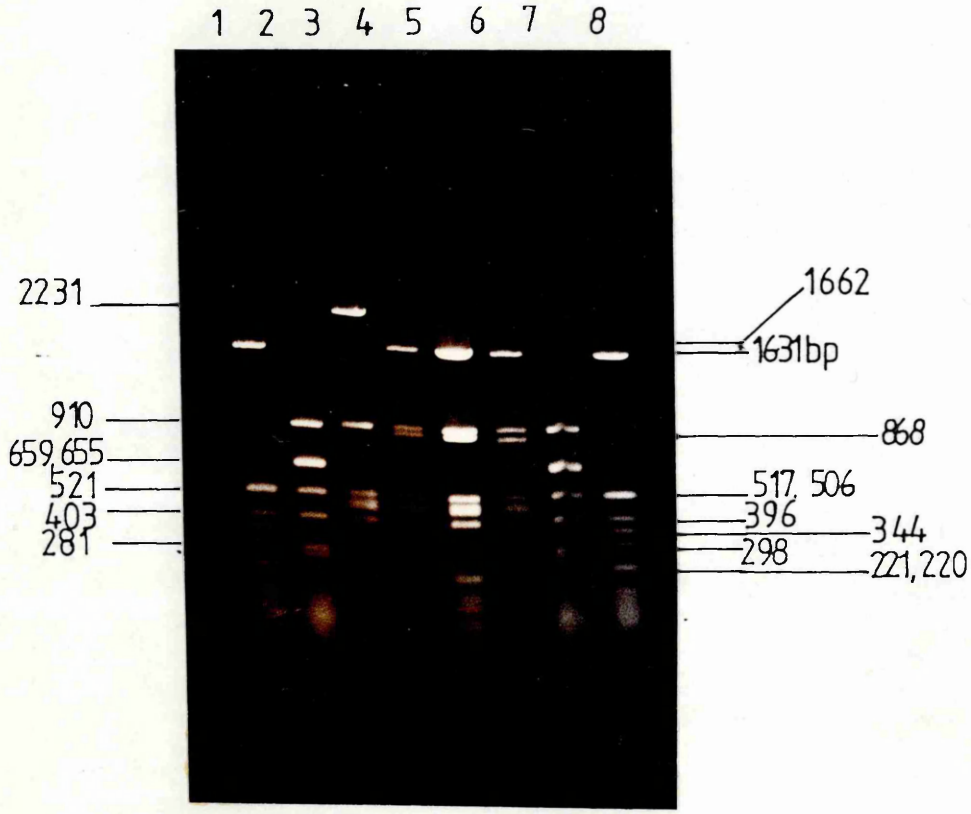


Figure 4.15A Restriction Analysis of pCJ107. 1% horizontal agarose gel showing a HinfI digest of pCJ107. The fragments produced are compatible with the par fragment cloned into the HincII site in the polylinker.

(1) pBR322 HinFI
(2) pBR322 AluI
(3) pKS501 HinFI
(4) pCJ107 HinFI
(5) " - "
(6) " "
(7) pBR322 AluI
(8) pBR322 HinFI

Figure 4.15 B

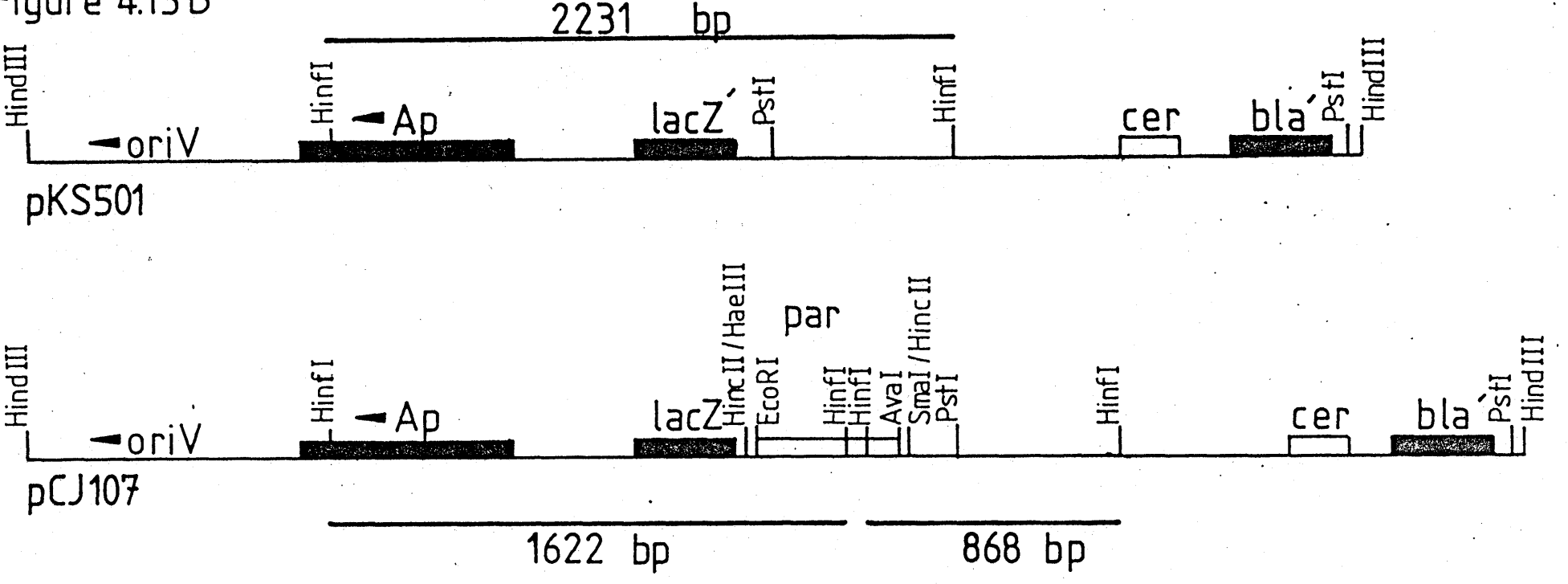


Figure 4.15B Linear Maps of pKS501 and pCJ107. The sizes of the relevant HinfI fragments in each construct are shown and agree with the restriction patterns obtained in Figure 4.15A.

plasmid derivatives which could selectively "amplify" particular regions of the plasmid via "unequal crossing-over" were used. The plasmid pKS501 can undergo inter-molecular "unequal crossing-over" in a recombination proficient host to amplify the replication origin containing region of the plasmid. Figure 4.14 shows the stucture of the amplifying vector. Upon introduction into JC8679, in addition to multimer formation, intermolecular recombination can occur between the 450bp of the bla gene end in the polylinker and the pUC9 bla gene. This produces a "pseudo-dimer" which has 2 origins, 2 bla genes and 2 cer sites. Site-specific recombination between the directly repeated cer sites resolves the "pseudo-dimer" into a plasmid which is dimeric only in the origin containing region of the molecule. The rest of the molecule is unaltered. A deletion product is produced but is not visible on gels since it lacks a replication origin and therefore cannot replicate.

To produce plasmid molecules which have an equivalent number of replication origins to dimers and trimers but only one par region, a SmaI-HaeIII fragment containing par from pCJ122 was cloned into the unique HincII site in the pKS501 polylinker to produce pCJ107. In such a position, site-specific recombination between cer sites on "pseudo-multimers" produces molecules with one par region and 2 or more replication origins. The presence of the par region was confirmed by a HinfI digest. The 2231bp fragment which results from a site within the pUC9 part of the plasmid and the fragment carrying the cer region in the polylinker, is replaced by 868 and a 1662bp fragments as shown in figure 4.15A and B.

Plasmid DNA from the hyper-recombinogenic host JC8679 containing pCJ107 was run on a 0.7% low melting point agarose gel in order to isolate the "origin-amplified" forms. Bands between the 1^m and 2^m supercoils were excised and the DNA used to transform DS903 (Fig 4.16). Restriction analysis revealed that a 2 origin 1 par plasmid (pCJ114) and a 3 origin 1 par plasmid (pCJ115) had been isolated. Figure 4.17 shows the positions of the polylinker sites on pCJ107 in relation to the bla' and cer regions. Restriction sites to the left of the partial bla region should be amplified, while sites to the right of cer will not, due to the site-specific recombination event between the repeated cer regions on a "pseudo-multimer".

Since the polylinker is composed of unique restriction sites, analysis

Figure 4.16

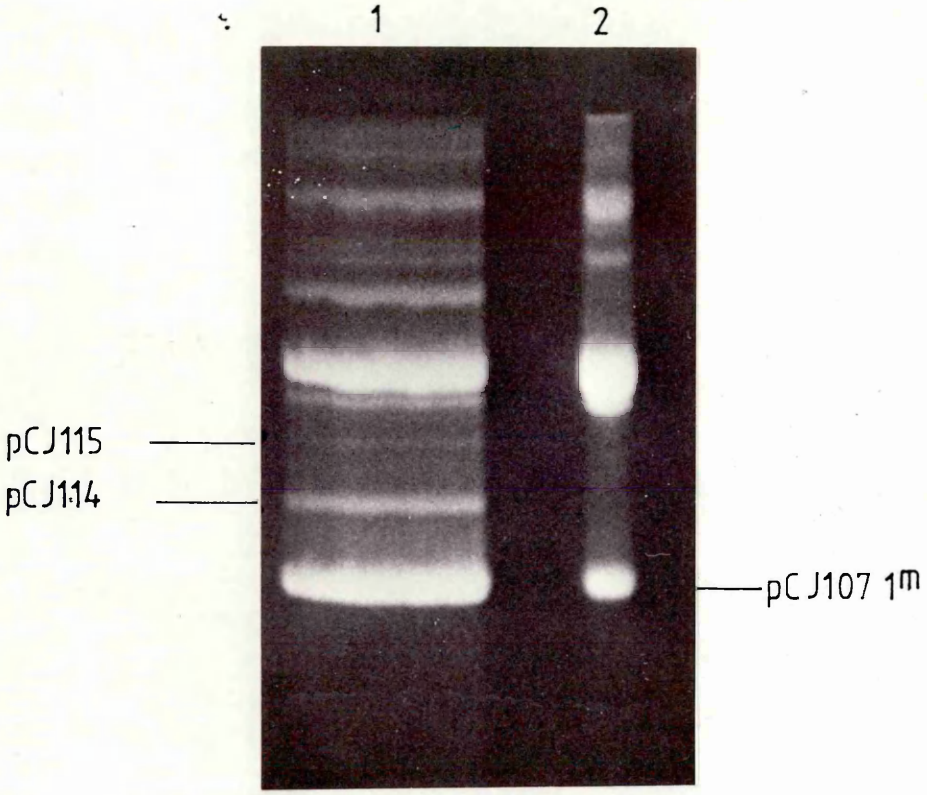


Figure 4.16 "Amplificants" of pCJ107. Additional bands running between the supercoils correspond to the amplified plasmid forms which differ by approximately 1.5kb.

(1) pCJ107 DNA from JC8679

(2) pCJ107 1^{m}

Figure 4.17

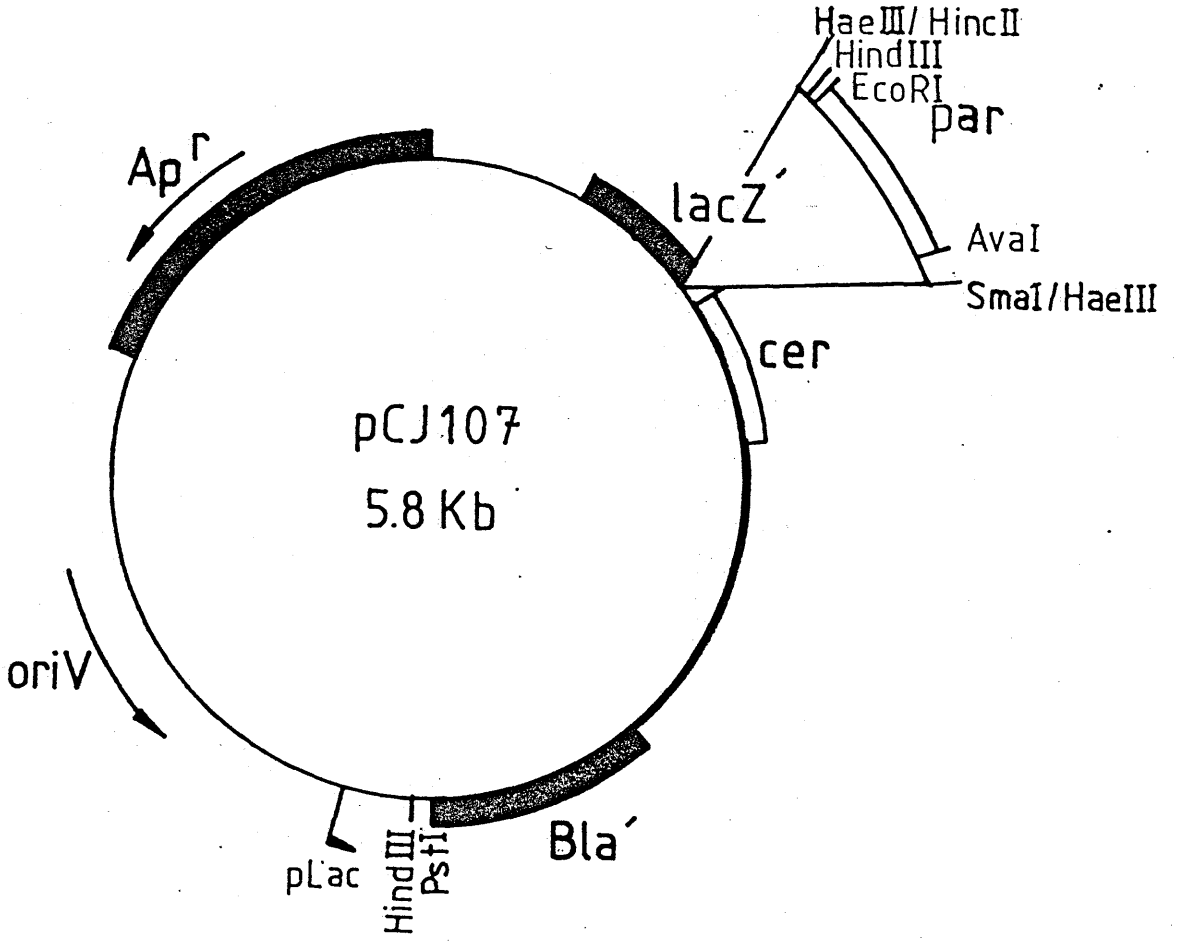


Figure 4.17 Schematic Diagram of pCJ107. The location of the polylinker sites with respect to cer are shown. Enzyme sites to the left of bla' will be duplicated in plasmids which are amplified for the origin region. Enzyme sites to the right however, will remain unique due to recombination between cer sites (see Fig 4.16).

Figure 4.18

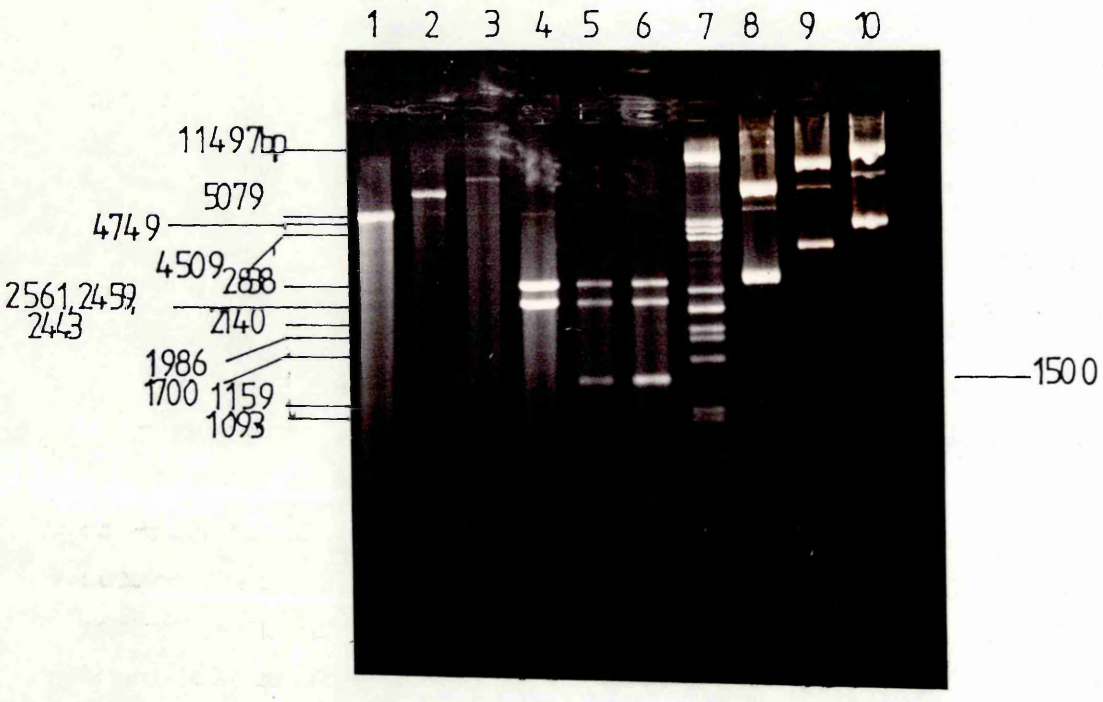


Figure 4.18 Demonstration of Unique and Duplicated Restriction Enzyme Sites in pCJ114 and pCJ115. Tracks contain the following:

(1) pCJ107 BamHI
(2) pCJ114 BamHI
(3) pCJ115 BamHI
(4) pCJ107 HindIII
(5) pCJ114 HindIII
(6) pCJ115 HindIII
(7) Lambda PstI
(8) pCJ107 s/c
(9) pCJ114 s/c
(10) pCJ115 s/c

of the "amplified" plasmids was simplified by using sites on either side of the bla' region and cer to demonstrate the region of the plasmid which had been amplified. A HindIII site lies to the left of bla' in the polylinker. Normally this site would be unique in pUC vectors, however, upon cloning par from pCJ122 via a HaeIII-SmaI digest, a second HindIII site from the pUC8 polylinker was introduced into pCJ107 (Fig.4.17). Digestion of pCJ107 by HindIII produced two fragments of approximately 2.7 and 3kb while digestion of the amplified forms pCJ114 and pCJ115 produced an extra fragment of 1.5kb. This corresponds to the size of the amplified replication origin containing region of pCJ114 and pCJ115.

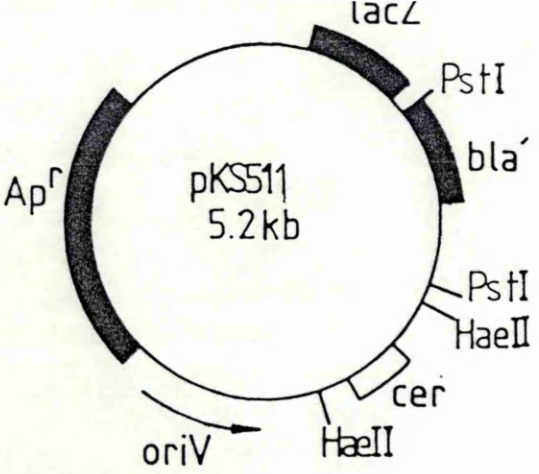
A unique BamHI site resides to the right of cer in pCJ107. Restricting pCJ107, pCJ114 and pCJ115 with this enzyme resulted in the linearization of all three plasmids (Fig.4.18). This demonstrated that the region of the plasmid to the right of the cer fragment had not been amplified and consequently the multi-origin plasmids pCJ114 and pCJ115 had only one par region.

4.8.2 CONSTRUCTION OF ONE ORIGIN, MULTIPLE PAR CONTAINING PLASMIDS.

It was possible to construct another set of plasmids similar to pCJ107, pCJ114 and pCJ115 which instead possessed one replication origin, but multiple par regions. This was accomplished using the plasmid pKS511. This plasmid was constructed from pLB04, a pUC9 based plasmid containing a 1.9kb HaeII cer containing fragment from ColE1, outside of the polylinker. Cloning a 703bp PstI fragment containing the end of a bla gene from a ColE1::Tn1 plasmid, involved restricting pLB04 with Pst1 which cuts the plasmid once in the polylinker and twice near the end of the cer fragment (D.Lee pers. comm.). The plasmid construct pKS511 is shown in figure 4.19A and it can be seen that the order of cer and the bla' region is reversed with respect to the origin amplifying plasmid pKS501 (Fig 4.14).

In order to amplify par regions using this plasmid, a 384bp par containing EcoRI fragment from pST2 was cloned into the EcoRI site in the polylinker to produce pCJ118. Figure 4.19B demonstrates the presence of the 384bp EcoRI par containing fragment in pCJ118 and figure 4.19C shows the position of par relative to cer and bla'.

To isolate pCJ118 derivative plasmids possessing one origin and



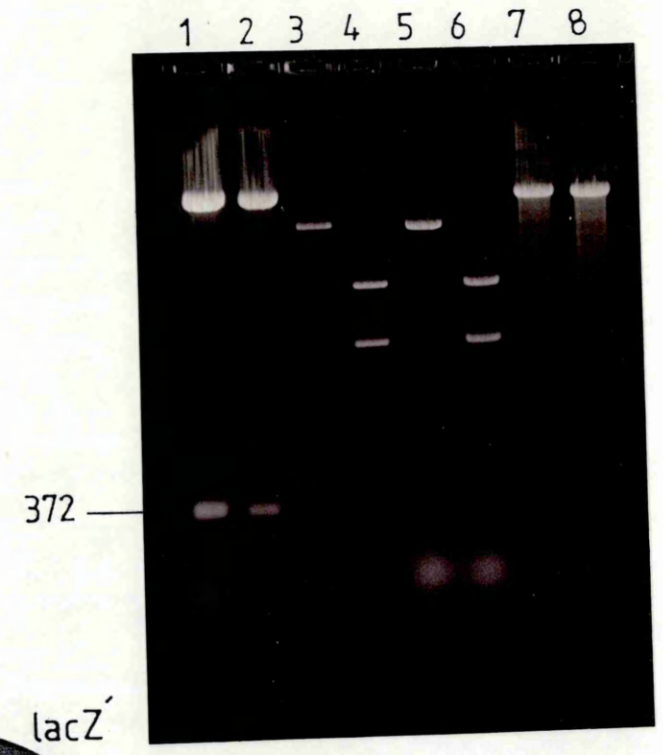


Figure 4.19C

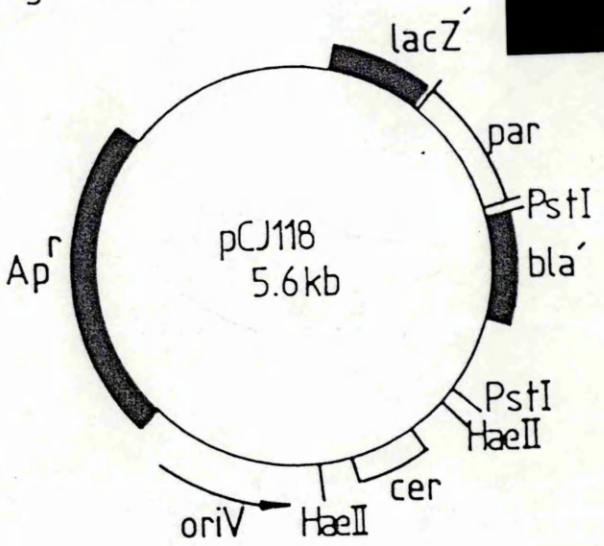


Figure 4.19A + B + C Restriction Analysis and Plasmid Maps of pKS511 and pCJ118. Figure 4.19A shows the location of cer and bla' in pKS511. Figure 4.19B is a 1% horizontal agarose gel and demonstrates the presence of the 372bp par fragment in pCJ118 and Figure 4.19C shows the location of par in pCJ118.

4.19B (1) pCJ118 EcoRI
(2) pCJ118 EcoRI/AvaI
(3) pST2 EcoRI
(4) pST2 EcoRI/AvaI
(5) pAT153 EcoRI
(6) pAT153 EcoRI/AvaI
(7) pKS511 EcoRI
(8) pKS511 EcoRI/AvaI

Figure 4.20

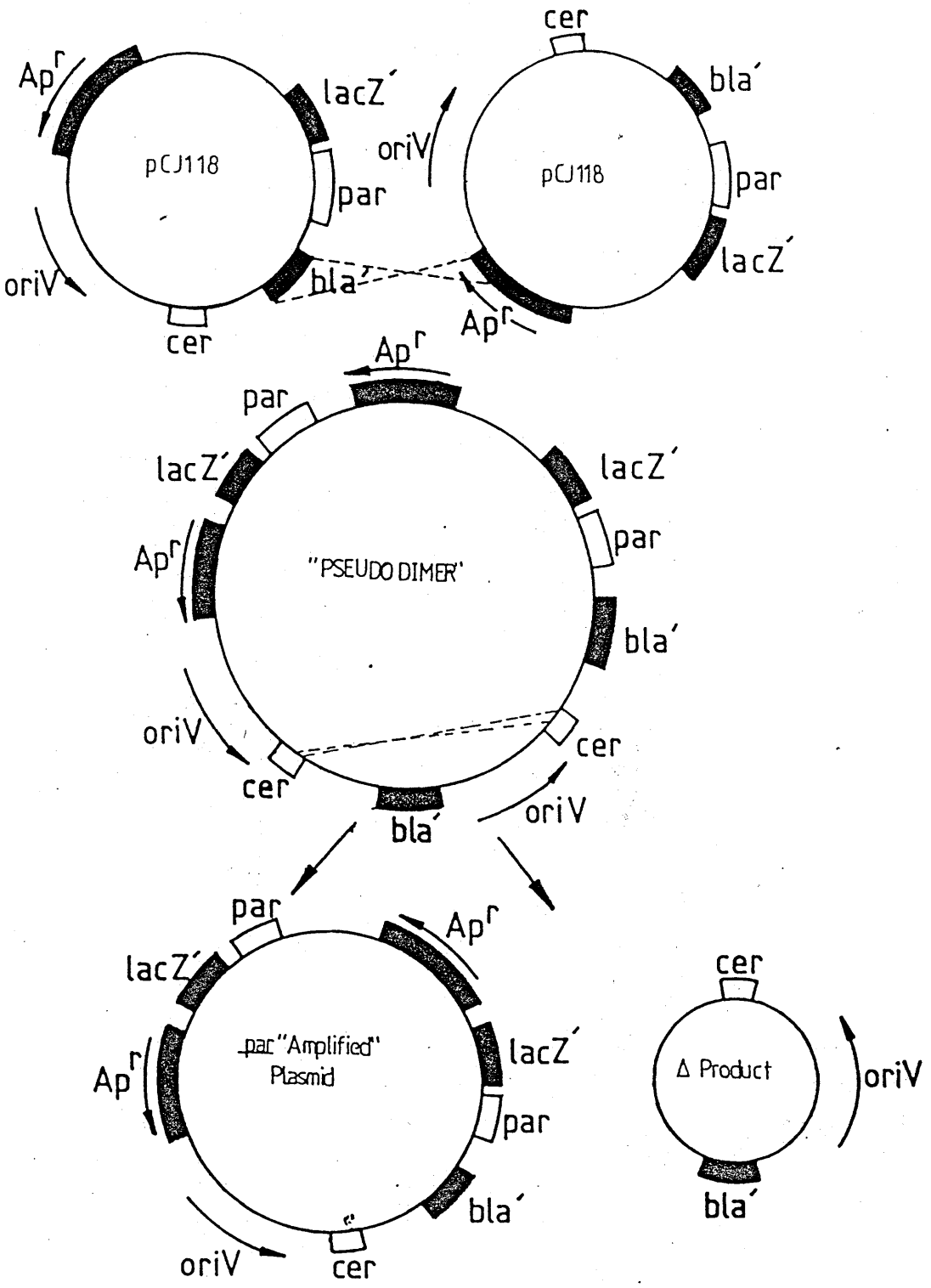


Figure 4.20 Schematic Diagram of Intermolecular Recombination Between pCJ118 Molecules Leading to the Formation of a Multiple par Plasmid and an Origin Containing Deletion Product.

multiple par regions, plasmid DNA was isolated from JC8679. The DNA bands on agarose gels between 1^m and 2^m supercoils were excised, gel purified and used to transform DS903. Because of the position of cer in pCJ118, unequal crossing over to produce a "pseudo-multimer", followed by site-specific recombination between directly repeated cer sites, results in the "monomerization" of the origin containing region of the plasmid. The multiple par region and multiple bla genes are not affected by recombination at cer. Consequently, molecules possessing 1 origin and multiple par regions are produced (Fig 4.20). A deletion product is obtained via the recombination events at cer. In contrast to the "origin-amplifying" vector pKS501, this deletion product can be visualized on agarose gels because it possesses a replication origin and can therefore replicate.

Since ampicillin resistance genes are also amplified in pCJ118 derivatives, it was possible to select for transformants having multiple par regions by plating onto cephaloridine at concentrations (55, 60 and 70ug/ml) at which the parent plasmid pCJ118 cannot grow. Colonies surviving on these concentrations were analysed by single colony gel analysis, which revealed the presence of the amplified forms pCJ119 and pCJ120 which were predicted to possess 2 and 3 par regions respectively (Fig.4.21A).

To confirm that the par containing region of the plasmid had been amplified, restriction digests were performed. Figure 4.21B demonstrates that both pCJ119 and pCJ120 have extra BamHI sites compared with the starting plasmid pCJ118. The "extra" band visible in tracks 2 and 3 corresponds to a BamHI fragment of 2.3kb which indicates that the intervening region between bla' and the end of the functional bla gene has been amplified. This, in addition to the calculated molecular sizes of the super-coiled plasmids, indicates that pCJ119 and pCJ120 have 2 and 3 par regions respectively. In contrast, resriction by the unique cutting enzyme MluI demonstrated that the region of the plasmid to the left of the bla' region i.e. the replication origin containing region, remains monomeric.

It was now possible to distinguish between the two possibilities which could explain the instability of multimeric par containing plasmids. (1). Do multiple par regions on a one origin plasmid disrupt par function? (2). Can one par region function in a multi-origin plasmid?

Figure 4.22 shows that the starting plasmids pKS501 and pKS511 and

Strain	Plasmid	Size (kb)	Calculated Copy Number			% Standard Deviation
			(a)	(b)	(c)	
DS903	pKS501	5.4	86	+/-11.3	3	13%
DS903	pCJ107	5.8	92	+/-12.2	3	13%
DS903	pCJ114	7.3	71	+/- 8.0	3	11%
DS903	pCJ115	8.8	57	+/- 8.6	3	15%
DS903	pKS511	4.9	92	+/-11.0	3	12%
DS903	pCJ118	5.3	94	+/-18.8	3	20%
DS903	pCJ119	7.6	103	+/-15.2	3	15%
DS903	pCJ120	9.9	97	+/-13.6	3	14%

Table 4.3 Calculated Copy Numbers from *in vivo* Labelling Data. Plasmid copy numbers were calculated per genome equivalent based on a figure of 3.8×10^3kb for the size of a non-replicating *E.coli* chromosome. The calculated copy numbers per genome equivalent are given in column (a), the standard deviations in column (b) and the number of independent trials in column (c). The copy number calculation was as follows:

$$\text{Copy Number} = \frac{\dfrac{\text{cpm plasmid}}{\text{cpm chromosome}} \times \text{Size } \textit{E.coli} \text{ Chromosome (kb)}}{\text{Size of Plasmid (kb)}}$$

Figure 4.22

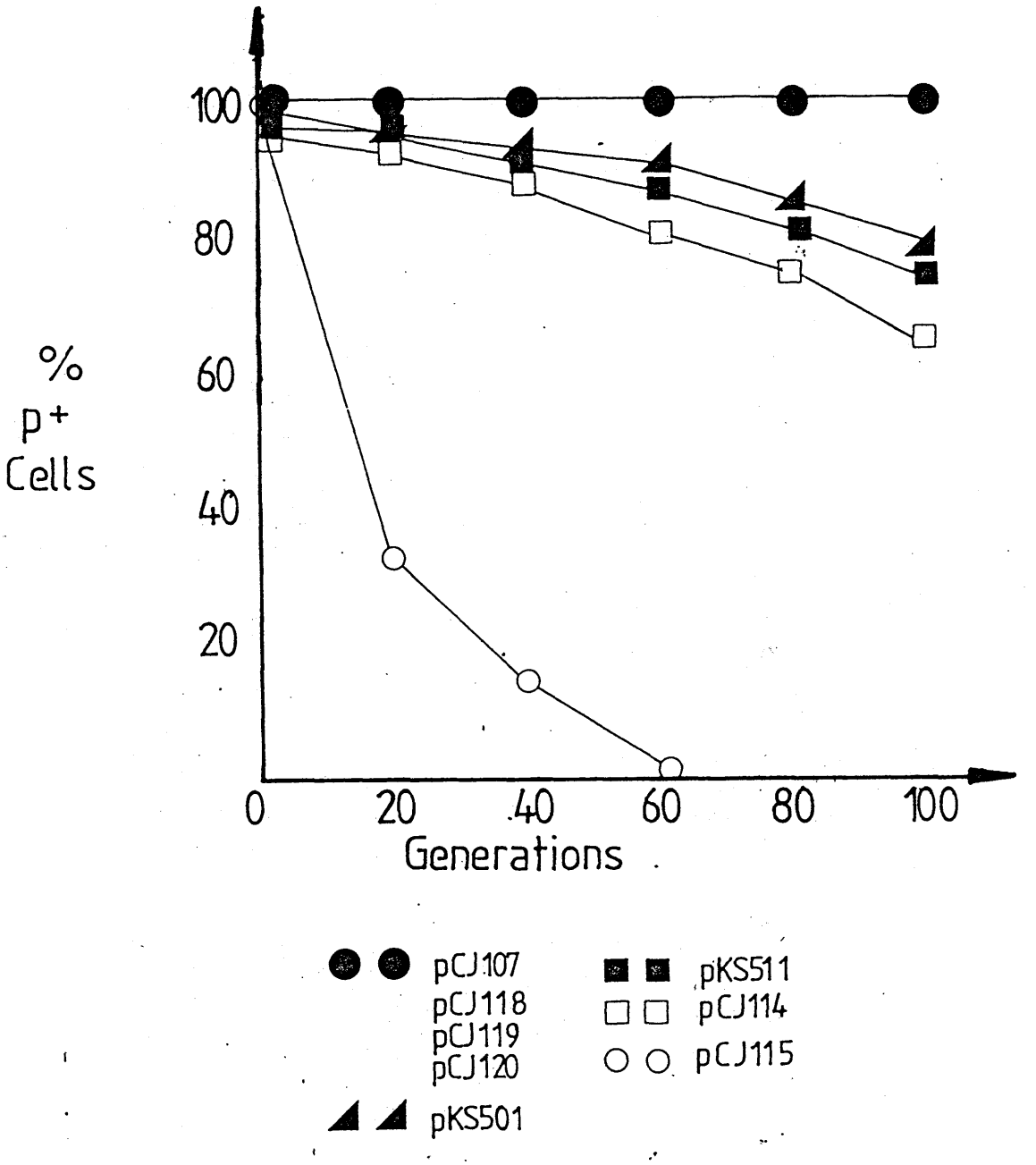


Figure 4.22 Stability Comparison of Multiple origin single par region plasmids and single origin, multiple par plasmids. The mean segregation frequencies for each plasmid are given below:

DS903	pKS501	2.4×10^{-3}	per cell per generation
"	pCJ107	$<10^{-5}$	" " " "
"	pCJ114	3.4×10^{-3}	" " " "
"	pCJ115	9.3×10^{-3}	" " " "
"	pKS511	2.3×10^{-3}	" " " "
"	pCJ118	$<10^{-5}$	" " " "
"	pCJ119	$<10^{-5}$	" " " "
"	pCJ120	$<10^{-5}$	" " " "

both are unstable. Plasmids pCJ107 and pCJ118 both possess one origin and one *par* region and are fully stable. This indicates that *par* is functioning in these plasmids. pCJ114 and pCJ115 possess 2 and 3 origins respectively but only one *par* region. Both are unstable and the 3 origin plasmid has a higher segregation frequency than the 2 origin plasmid. In contrast, the multi-*par*, one replication origin plasmids are all fully stable. The copy numbers of all 8 plasmids are shown in table 4.3. The one origin plasmids pKS511, PCJ118, pCJ119 and pCJ120 have very similar copy numbers, while the copy numbers of the multi-origin plasmids, decrease with increasing origin number, as predicted by the "origin-counting" hypothesis discussed in Chapter 3.

If *par* is functional, the instability of the multi-origin plasmids cannot be due to the reduction in copy number, since plasmids possessing a *par* locus are theoretically stable down to a copy number of 2 per cell. This implies that *par* fails to function in these plasmids, but can in the one-origin, multi-*par* plasmids pCJ119 and pCJ120. Multiple *par* sites within one molecule therefore do not interfere with *par* function, however, *par* mediated active partitioning cannot occur when plasmids possess multiple replication origins.

CONCLUDING REMARKS

A 372bp *cis*-acting DNA region designated *par* is required for stable inheritance of pSC101 (Meacock and Cohen 1980). Its *cis*-acting nature, absence of coding potential and dissimilarity to *cer*, indicate that it is likely to be a site which accomplishes even distribution of plasmids to daughter cells by interacting with host encoded components of the partitioning system and it may be analogous to the centromere of eukaryotic chromosomes.

Meacock and Cohen (1980) demonstrated that *par* could fully stabilize an unrelated multicopy plasmid pACYC184. This suggested that *par* was not replicon specific and further studies indicated that orientation and proximity to the origin in pSC101 did not affect *par* function.

The results in this chapter demonstrate that *par* cannot function in certain plasmids. The observation that pCJ101, but not the similar plasmid pST2 was stabilized by *par*, suggested that in contrast to Meacock and Cohen's conclusions, location and proximity to the origin may be important for *par* function in some plasmids.

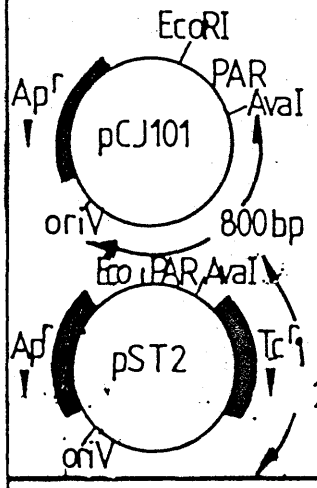
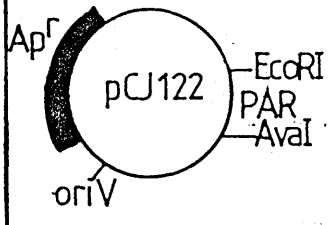
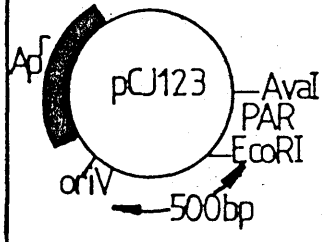
PLASMID	STABILITY				
	1m	2m	3m	6m	8m?
 Also pWX9	(29) ✓	(17) ✓	(14) ✓	×	××
	(26) ✓	(16) ×	(12) ××		
	(125) ✓	(93) ✓	(70) ××		
	(115) ✓	(91) ×	(78) ××		
pCJ107 1origin 1par	(92) ✓	—	—		
pCJ114 2 " 1 "	—	(71) ×	—		
pCJ115 3 " 1 "	—	—	(57) ××		
pCJ118 1 " 1 "	(94) ✓	—	—		
pCJ119 1 " 2"	(103) ✓	—	—		
pCJ120 1 " 3 "	(97) ✓	—	—		

Table 4.4 Summary of the Copy Number and Stability Properties of Several par Containing Plasmids. The distance between the replication origin and par in each construct is shown. Stability is denoted by a tick, instability by a cross and the number of crosses indicates the severity of the instability. Copy number values for each plasmid are shown in brackets.

The stability and copy numbers of several *par* containing plasmid constructs were analysed and the results are tabulated in Table 4.4. A rational explanation of these data requires that the plasmid constructs pCJ101, pWX9 and pST2 be considered separately from the pUC8 and 9 derivatives, since although pUC plasmids are derivatives of pBR322, the absence of some sequences (tetracycline resistance) and the presence of others (*lac* sequences), will alter the transcriptional environment. Since transcriptional readthrough may affect *par* function, a direct comparison between the pAT153 derivatives pCJ101, pWX9 and pST2 and the pUC derivatives cannot be made.

The monomers of pCJ101 and pST2 were stably maintained, but multimeric forms of pST2 were not. Since pAT153 (the parent plasmid) is also fully stable, the pST2 results alone could not be interpreted to mean that *par* was unable to stabilize multimeric molecules since it was possible that *par* failed to function at all in this plasmid construct.

Three main factors may account for the stability of pCJ101 2^m and 3^m species and the instability of the corresponding forms of pST2. Firstly, the proximity of *par* to the replication origin in both constructs differs greatly, since in pCJ101 the distance is 800bp but in pST2 is 2.5kb. In nature, *par* is located approximately 200bp from the pSC101 replication origin and is one of the first regions to be replicated. It has been suggested that *par* is only active when newly replicated, however, by observing the segregation kinetics of temperature sensitive plasmid replication mutants at the non-permissive temperature, Tucker *et al* (1984) showed that non replicating par^+ plasmids were actively partitioned. Secondly, the transcriptional environment in pST2 differs from that of pCJ101 and pWX9 due to the presence of the "anti-tet" promoter and it is feasible that transcription traversing the *par* region in pST2 accounts for its lack of function in this plasmid. However, computer analysis of the pSC101*par* sequence (Fig.4.9) led to the identification of a putative *rho*-independent transcription terminator (Miller *et al* 1983). The terminator has runs of T residues either side of the the G-C rich stem and therefore should function in either orientation (Adhya and Gottesman 1978). Tucker *et al* (1984) however, could only demonstrate terminator activity in one orientation by assaying tetracycline resistance in a terminator-probe vector. If this terminator does function in only one orientation, transcription from the "anti-tet" promoter in pST2 will

Figure 4.23

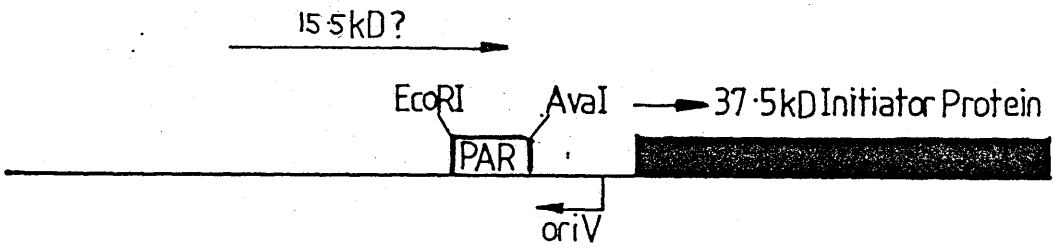


figure 4.23 Schematic Diagram of the Organization of the par Containing Region of pSC101. An open-reading frame capable of coding for a 15.5kD protein terminates within the 372bp par region. par is replicated prior to other regions on the plasmid molecule due to its location on the plasmid.

proceed unhindered through par and this may explain the instability of this plasmid.

The complete pSC101 sequence is available (Bernardi and Bernardi 1984) and in an effort to determine the nature of the local environment around par in pSC101, the sequence of the replication origin region was subjected to computer analysis. A potential protein coding sequence of 15.5kD capacity is counter-transcribed and located downstream of the replication origin (Fig 4.23). par resides between this putative protein and the replication origin and part of the 372bp EcoRI-AvaI par containing fragment overlaps with the 3' end of the protein coding region. This overlap suggests that the first 160bp of the EcoRI-AvaI fragment is not required for par activity. Limited deletion analysis (Kalla and Gustafsson 1984) has demonstrated that the minimal par region includes bases 69-246 of the 372bp EcoRI-AvaI fragment (Fig.4.9). Further study however, may reveal that the active par region can be further delimited. The T residues of the functional par terminator lie 45 bases from the stop codon for the 15.5kd gene. This suggests, but does not demonstrate conclusively, that the terminator within par is not connected with the stability function of this region but rather functions in nature to prevent counter-transcription running into the replication origin of pSC101. Transcriptional readthrough into par however, has been implicated to explain partial par activity in pBR322trp$^+$par$^+$ constructs (Skogman et al 1983).

Finally, it is possible that par does not function to stabilize multimers of pCJ101 and pWX9. In these constructs, the Tc^r region of pAT153 and pBR322 was removed (1424bp) and was replaced by the pSC101 par region. The stability of pCJ101 and pWX9 2^m and 3^m were compared with their pAT153 and pBR322 counterparts respectively and not with tetracycline resistance region deletion derivatives. Constructing such plasmids would involve double digestion of pAT153 and pBR322 with EcoRI and AvaI, gel purification of the large fragment of each plasmid, followed by blunt ending with Klenow in the presence of all 4 dXTP's and ligation to produce Ap^{r}Tc^s deletion derivatives. Subsequent stability testing on the multimeric forms of these plasmids would determine if they were stably maintained without the par region. The observation that both a 6^m and a possible 8^m of pCJ101 were unstable supports the view that par cannot stabilize multimers and indicates that the stability of the 2^m and 3^m of this plasmid may indeed be due to factors

other than par.

The second category in Table 4.4 are pUC8 and 9 derivatives. pCJ122 and pCJ123 contain the par fragment in two orientations with respect to the lac promoter and the replication origin, which is 500bp distant. The observation that pCJ122 2^m is fully stable while pCJ123 2^m is not, may reflect a preferential orientation with respect to the replication origin or to strong transcriptional readthrough. It may be significant that in nature, the orientation of par with respect to direction of oriV replication is identical to that in pCJ122 but not in pCJ123. The 3^m species of both plasmids are unstable, which demonstrated that par does not function in these plasmid forms.

The use of vectors to selectively amplify regions of plasmids, facilitated the construction of molecules possessing either multiple origins with one par region, or conversely, multiple par regions with only one replication origin. The stability of the high copy number, multiple par containing plasmids pCJ119 and pCJ120 suggests that any cellular component which par may bind to during active partitioning, is not present in limiting amounts and excess par regions do not "clog-up" the putative par binding sites.

The instability of the multiple origin constructs pCJ114 and pCJ115, is in accord with the previous observations that par cannot stabilize multimers of ColE1-like plasmids. It would be of interest to determine whether par could stabilize multimers of pSC101, if indeed they exist, since it is possible that this natural plasmid possesses a system similar to ColE1 which resolves multimers to monomers.

Tucker et al (1984) demonstrated that Cmp$^-$ pSC101 plasmids show reduced transformation frequencies of cells already possessing resident pSC101par$^+$ plasmids. This reduction greatly exceeds that seen due to normal incompatibility and is not observed when the resident plasmid is par$^+$ pACYC184. This implies that the Cmp$^-$ phenotype is probably associated with the replication control system of the plasmid. Perhaps deletion of one partition related segment reduces the affinity of par for its binding site. If the replication enzymes are adjacent to this site, par$^+$ plasmids may be preferentially replicated rather than Cmp$^-$ plasmids. Furthermore, the stable co-existence of a Cmp$^-$ pSC101 plasmid with a pACYC184 par$^+$ derivative, indicates that there may be different replication sites for unrelated plasmids. Gustafsson et al (1983) demonstrated binding between the par region of pSC101 and the

outer membrane of E.coli. It has already been demonstrated that certain outer membrane components have an affinity for DNA and indeed a specific high affinity binding between the oriC region of the E.coli chromosome and the outer membrane has been shown to occur (Hendrickson et al 1982). Such an association was predicted by Jacob et al (1963) to explain regulation of DNA replication and segregation of the bacterial chromosome.

The Tucker data, in addition to the demonstration of membrane binding by par suggest a model for par function. It is feasible that the par region of pSC101 binds to a specific membrane component which may be adjacent to membrane bound replication enzymes. These replication sites may differ for different plasmids, since pSC101 unlike most plasmids, requires the host encoded dnaA gene for replication (Hasanuma et al 1977). Plasmids completely lacking a par region, or having 2 out of the 3 partition related segments deleted, have similar copy numbers to the wild-type plasmid but segregate at high frequency. Thus, binding via par to the putative membrane site is not a pre-requisite for replication but may ensure that each individual plasmid copy is accurately partitioned at cell division.

The inability of par to stabilize multimers of ColE1-derived plasmids is not easily understood, but may be a result of several factors acting in concert. It cannot be explained by the reduced copy numbers of multimers, since theoretically par should stabilize a plasmid with a copy number of just 2 at cell division. Perhaps there are sites on the membrane to which ColE1-derivatives become attached at oriV in order to replicate. Multimeric molecules unlike monomers will form multiple attachments which could prevent a physical interaction between par and its binding site on the outer membrane.

Contrary to the conclusions of Meacock and Cohen (1980), the results from this chapter demonstrate that par cannot fully stabilize all plasmids. Both proximity to the replication origin and transcriptional environment may affect par function and therefore if par is to be considered as an efficient method of stabilizing plasmids, these factors will have to be borne in mind during the construction of stable vectors. In addition, recombination deficient host cells or the cer region of ColE1 should be employed in order to keep multimers to a minimum.

CHAPTER 5

THE EFFECT OF *par* ON THE "FITNESS" OF HOST CELLS.

Despite intensive study of pSC101 *par*, no analysis of the effect of *par* on the "fitness" of host organisms has been performed. Therefore the formal possibility exists that *par* enhances the ability of a host cell to compete favourably with any plasmid-free cells which may arise. Indeed, there have been several reports in the literature of certain DNA elements IS50, Lambda, Mu, P1 and P2 conferring a growth advantage on host cells containing them (Biel and Hartl 1983; Hartl *et al* 1983; Edlin and Kudrna 1975; Edlin *et al* 1977; Lin *et al* 1977). Since initial data on pCJ101 stability indicated that *par* may function in this plasmid (Chapter 4, section 4.2), a study of its ability to compete in mixed culture with plasmid free cells was performed.

The study of competition between p^- and p^+ cells is difficult to perform in batch culture due to fluctuations in both nutrient availability and physiological state of the cells. These problems can be overcome by the use of continuous culture apparatus (chemostat). A chemostat consists of a culture vessel connected to a reservoir of sterile medium via a feeding system which admits medium to the vessel at a constant rate via a peristaltic pump. A syphon overflow driven by the same pump removes bacterial suspension from the apparatus at a rate equivalent to the incoming fresh medium. If the pump is set to deliver medium at a rate greater than the maximum specific growth rate (umax) of a particular bacterium, the culture will be "washed out" due to its inability to double at the rate dictated by the dilution rate "D". In practice, chosen dilution rates are lower than the umax of a bacterial species and the cell population increases until steady-state conditions prevail and the growth rate decreases to equal the dilution rate, due to exhaustion of a limiting nutrient.

The balance between the two processes, growth and "wash-out" will determine the net change in concentration of the bacteria.

$$\text{Increase} = \text{Growth} - \text{Output}$$

$$dx/dt = ux - Dx$$

$$dx/dt = x(u - D) \quad (1)$$

Where x = initial population size
D = dilution rate
u = growth rate constant

Only when u = D will the concentration of organisms remain constant with time.

Thus, a chemostat provides a constant environment in which cells can be maintained indefinitely in exponential phase, which permits the study of competition between plasmid-free and plasmid-containing organisms.

Competition between strains can occur for several reasons, one strain may produce a substance which interferes with the growth of the other organism e.g. colicin production from ColE1 containing cells inhibits the growth of plasmid-free cells. A more common reason for competition however, is simply that in batch culture they have different growth rates, or in chemostat culture they have different affinities for the growth limiting substrate at the particular dilution rate used (Tempest 1969).

RESULTS AND DISCUSSION.

5.1 Analysis of the Outcome of a Mixed Culture Experiment with Differentially Marked Plasmid-Free Host Strains.

Competition experiments between plasmid-containing and plasmid-free hosts are often complicated by the production of p^- segregants from the p^+ cells especially when dealing with a plasmid which is known to be unstable. This chapter discusses plasmids which are stably maintained in batch culture, however, to ensure that plasmid segregation did not affect the outcome of mixed culture experiments in continuous culture, differentially marked host strains were constructed. Their construction (discussed in chapter 6), involved the exchange of the *stp*^{r} marker of DS903 for *spc*^{r} via P1 generalised transduction, generating a Spc^r derivative of DS903.

To determine how each strain fared in mixed culture, two chemostats were separately inoculated with the Stp^r and Spc^r strains and grown into

Figure 5.1A

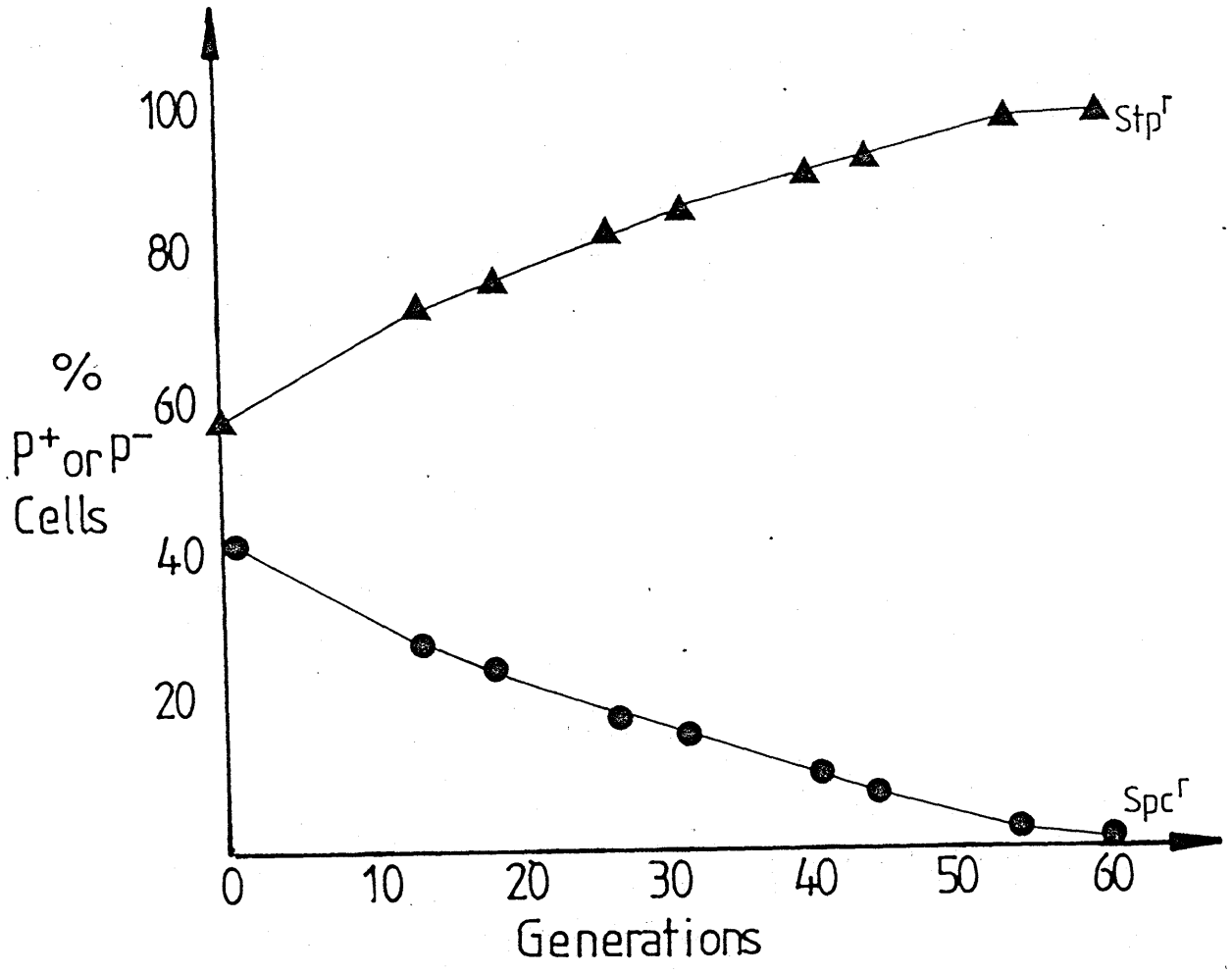


Figure 5.1A Competition Experiment Between Stp^r DS903 and Spc^r DS903.
Stp^r DS903 was resident and was challeged with Spc^r DS903 cells.

Figure 5.1B

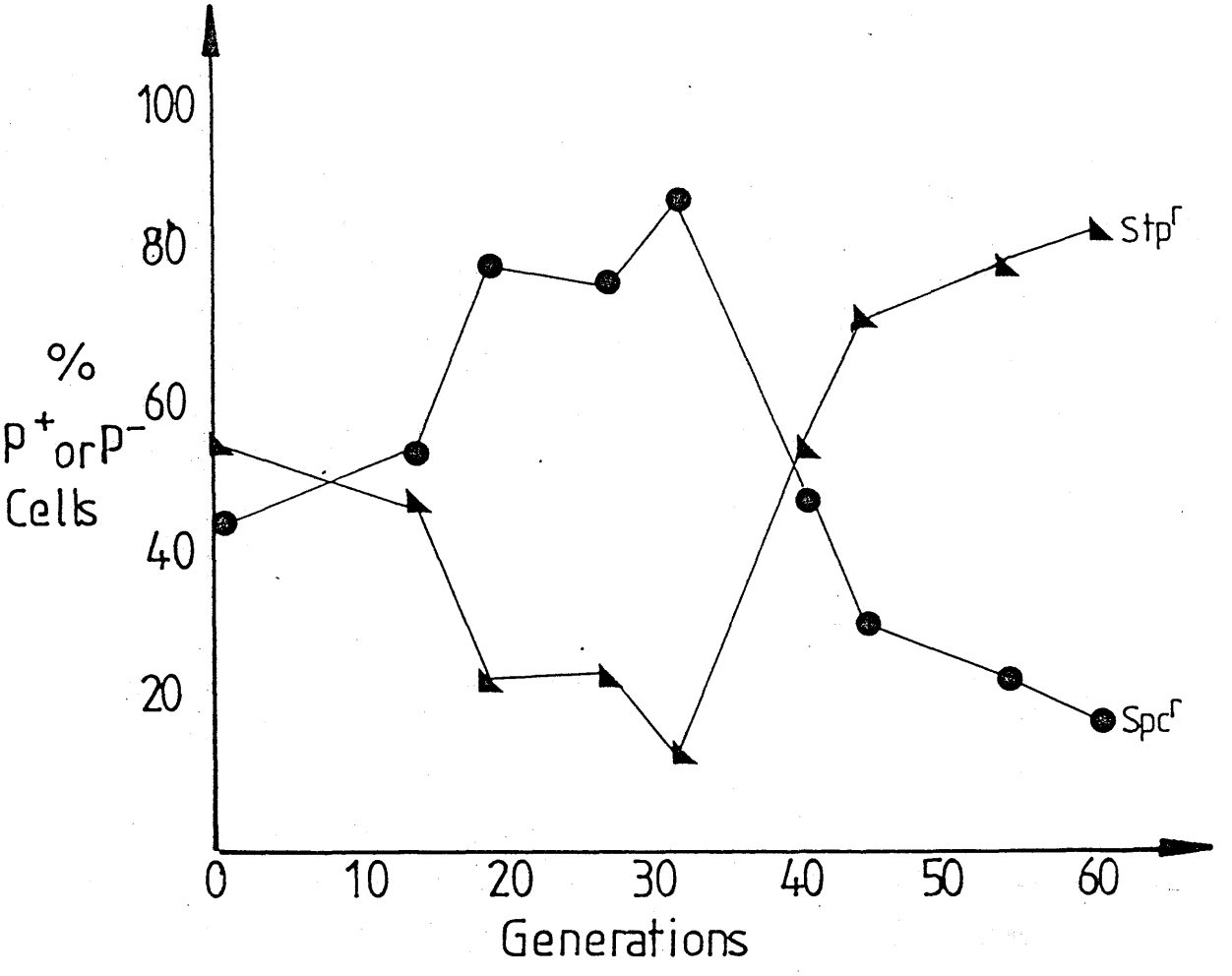


Figure 5.1B Competition Experiment Between Spc^r DS903 and Str^r DS903. Spc^r DS903 was resident and was challenged with Str^r DS903 cells.

late-log phase prior to switching on the media pumps and allowing the culture to become "continuous".

The dilution rate D, is related to culture volume and flow rate in the following manner:

$$D = f/V$$

The working volume of each chemostat was 600ml and D was set at $0.4hr^{-1}$ i.e. 240ml/hr. Because $D = 0.693/t_d$ where t_d is the division time, a dilution rate of this order will give a doubling time of 1.7 hours.

After several hours of continuous growth, 300ml of the culture was removed from each chemostat and added to the other vessel, thereby mixing the Spc^r and Stp^r DS903 bacteria. Samples were withdrawn soon after adequate mixing had occurred and subsequently every 5-7 generations. The results of the reciprocal experiments are shown in figures 5.1A and B. Figure 5.1A shows the outcome of the mixed culture experiment when the Stp^r species was resident in the chemostat prior to the addition of Spc^r DS903. Initially, Stp^r bacteria constituted almost 60% of the culture and subsequent generations led to this strain predominating. The result of the reciprocal experiment is shown in figure 5.1B and once again at the end the Stp^r stain has a competitive advantage over the Spc^r strain. However, in this experiment the resident Spc^r strain initially outgrew the Stp^r cells. In this early period the total cell count at each time point varied significantly, prior to stabilizing, indicating that some environmental parameter was not constant. This casts some doubt on the validity of the final outcome of this experiment. However, it is probable that the Stp^r strain does indeed have a growth advantage over Spc^r cells on the basis of the poor growth of the Spc^r strain in L-broth batch culture (data not shown). It was therefore not feasible to use this strain as a host for plasmids in mixed culture experiments since competition between p^+ and p^- cells, would be further complicated by competition occurring between the differentially marked Spc^r and Stp^r hosts.

Figure 5.2.A

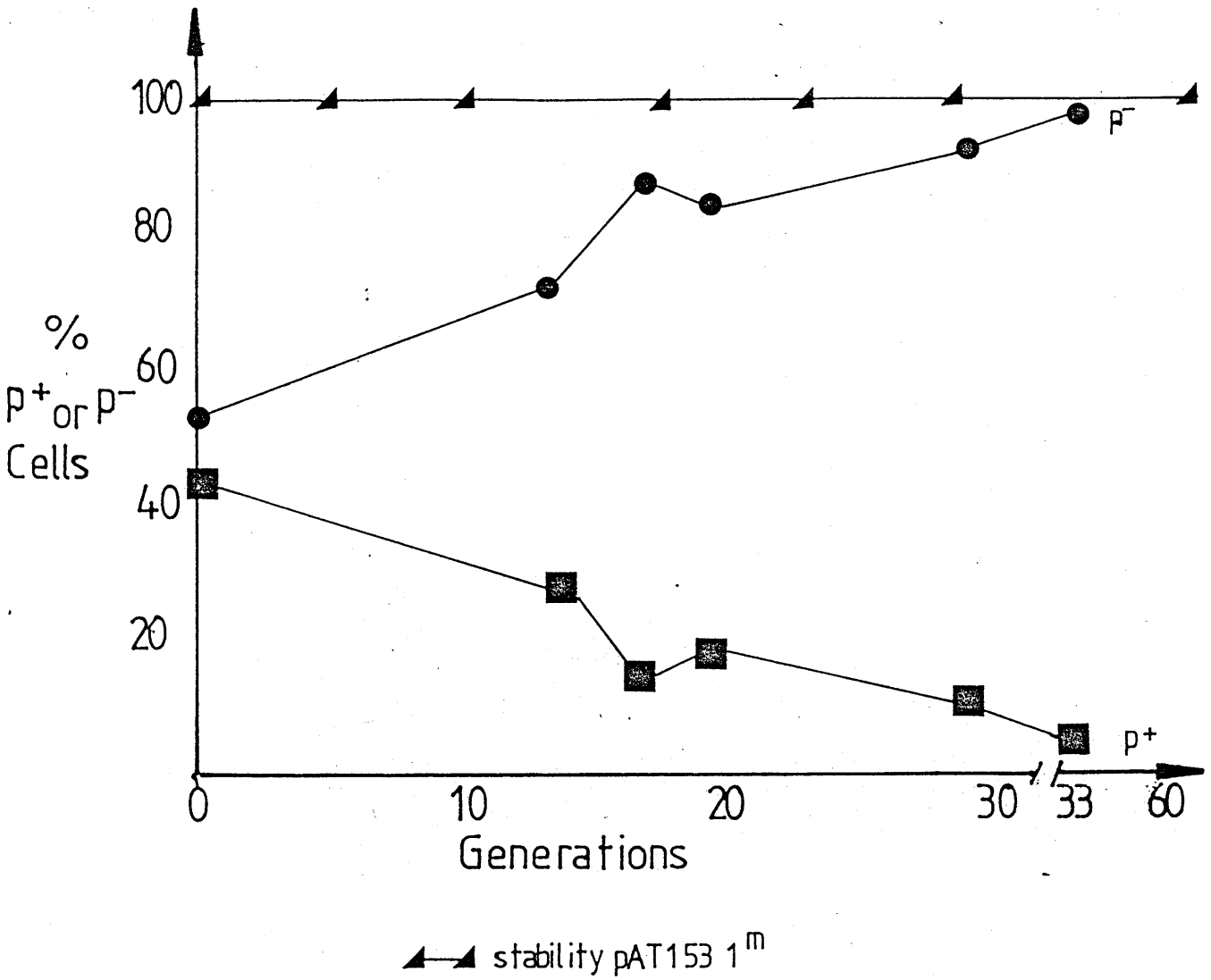


Figure 5.2A Competition Experiment Between pAT153 1^m containing DS903 cells and DS903 Plasmid Free Cells. The plasmid containing cells were resident prior to challenging with plasmid free DS903.

Figure 5.2B

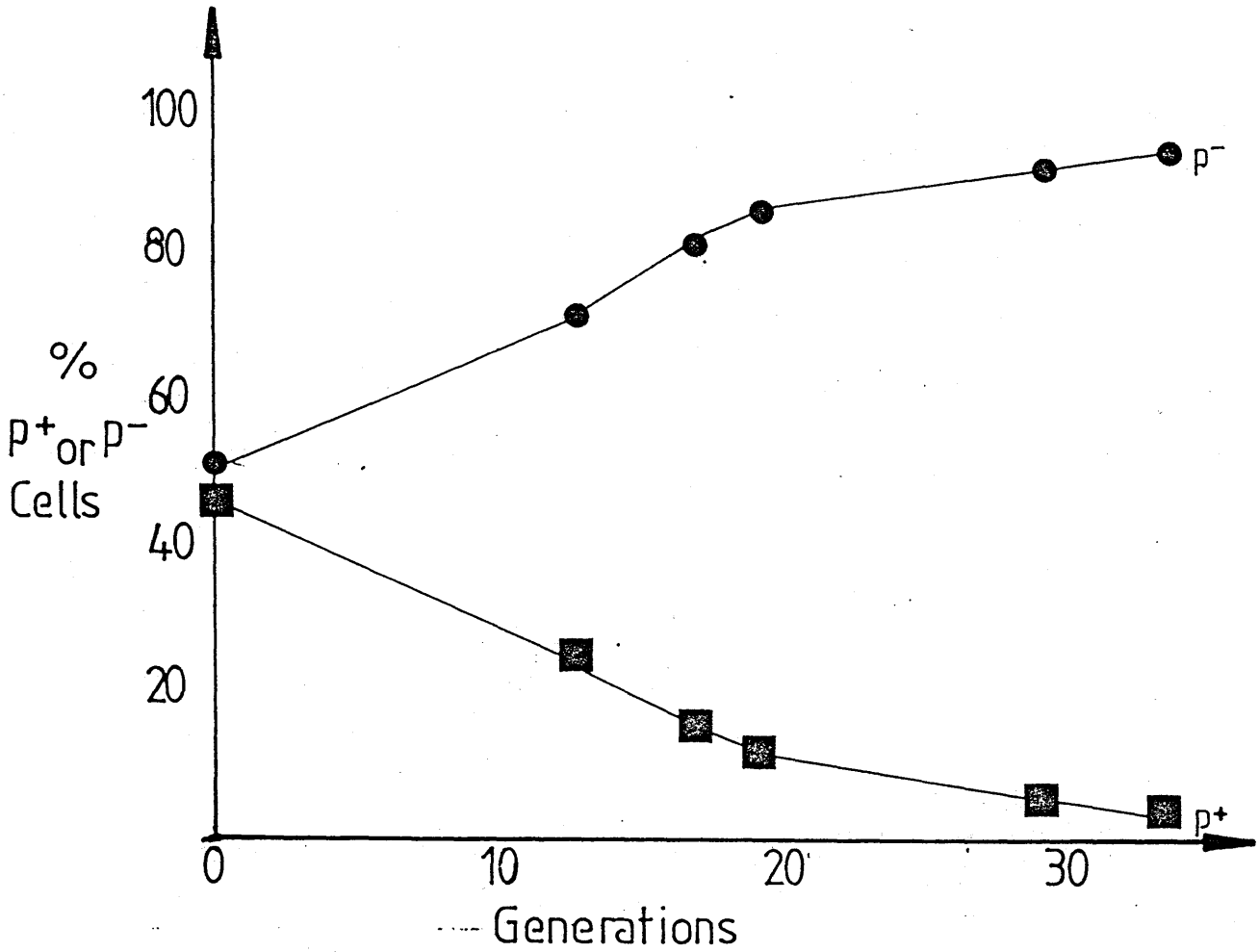


Figure 5.2B Competition experiment Between Plasmid Free DS903 and pAT153 1^{m} Containing Cells. The DS903 plasmid free cells were resident prior to challenging with the plasmid containing DS903.

Figure 5.3A

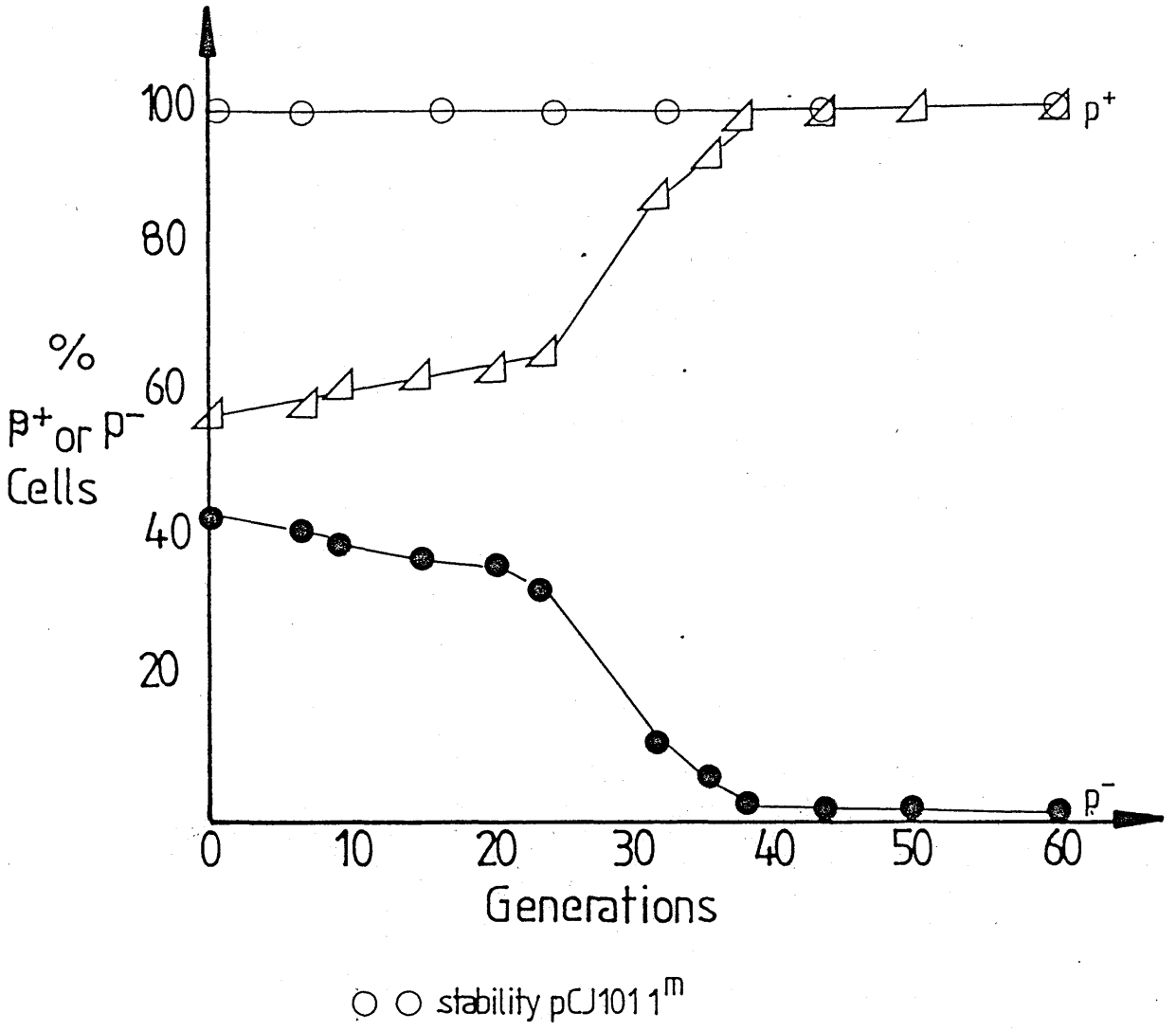


Figure 5.3A Competition Experiment Between pCJ101 1^m Containing DS903 and Plasmid Free DS903 Cells. The plasmid containing cells were resident prior to challenging with the plasmid free cells.

Figure 5.3B

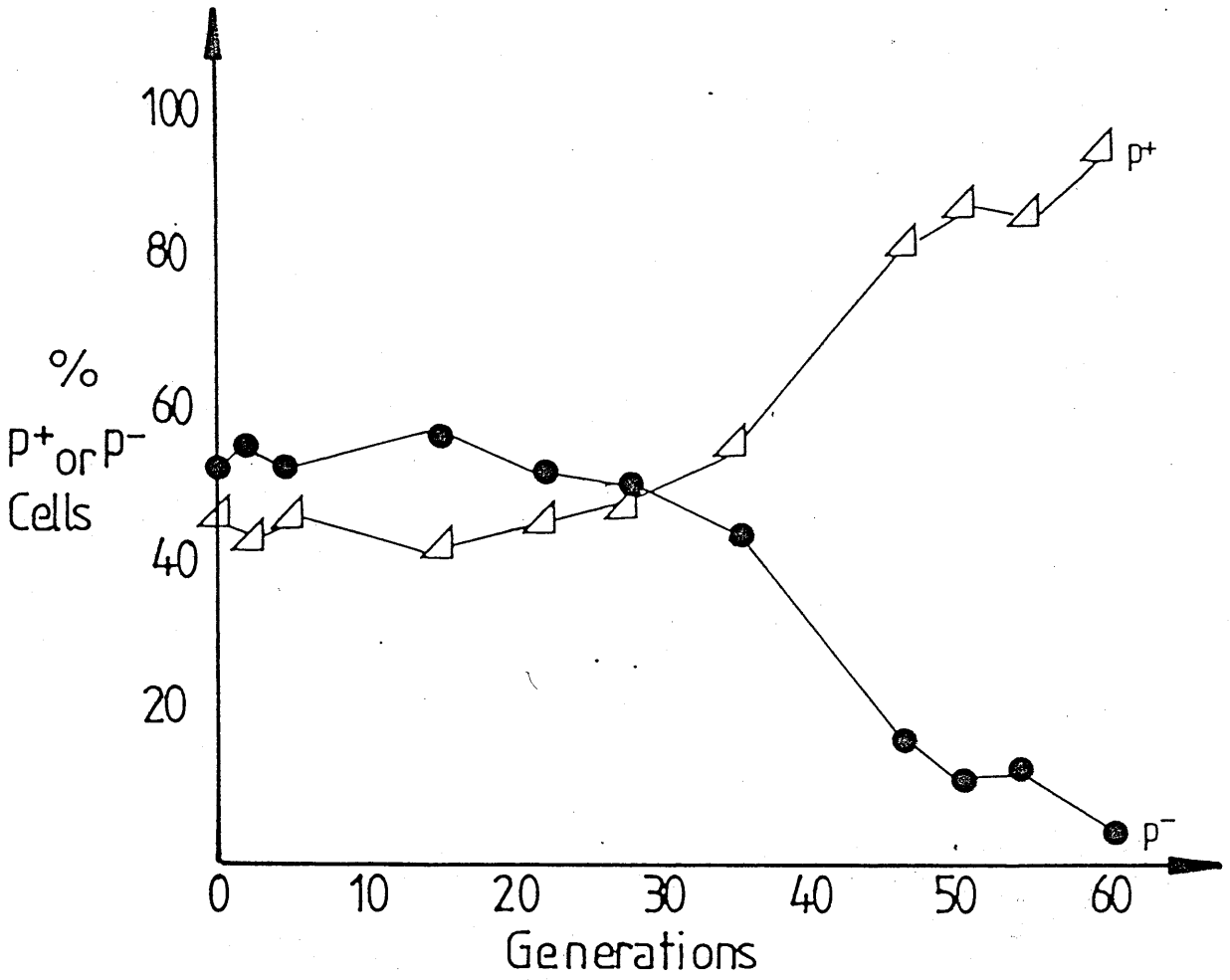


Figure 5.3B Competition Experiment Between Plasmid Free DS903 and pCJ101 Containing DS903 Cells. The plasmid free cells were resident prior to challenging with the plasmid containing cells.

5.2 Mixed Culture Experiments with DS903 pAT153 1^m Containing Cells and Isogenic Plasmid-Free Cells.

To determine the effect of pAT153 on host cells in competition with plasmid-free cells, mixed cultures were set up in the same manner as the above experiment. Samples were removed periodically, plated onto drug free isosensitest agar and the proportion of plasmid-free cells was determined using the B-lactamase plate assay for ampicillin resistance. The results of the reciprocal experiments are shown in figure 5.2A and B. In both cases, the plasmid-free cells rapidly outgrew the pAT153 1^m containing cells. Since it was not possible to use differentially marked host strains to determine whether segregation contributed to the take-over by p^- cells, the stability of pAT153 1^m in DS903 was examined under chemostat conditions for the same number of generations and was demonstrated to be stably maintained. Thus it appears that plasmid-free DS903 cells have a growth advantage over pAT153 1^m containing DS903 cells.

5.3 How Does pCJ101 Perform in Competition with the Plasmid-Free Isogenic Host?

Although there is good evidence that pSC101*par* is involved in plasmid partitioning (Tucker *et al* 1984), the formal possibility exists that *par* may enhance the ability of bacteria to compete with any plasmid-free cell which may arise in a culture. To test this, mixed culture experiments containing plasmid-free DS903 and DS903 pCJ101 cells were set up as before. The fate of the plasmid-containing organisms was monitored regularly for a period of 60 generations, using the B-lactamase plate assay and the results are shown in figure 5.3A and B. When a p^- inoculum was added such that it constituted 43% of the mixed culture, DS903 pCJ101 increased relative to the p^- cells. After 60 generations, pCJ101 containing cells constituted 99% of the culture, indicating that the plasmid containing cells had a competitive advantage over the plasmid-free DS903 cells.

The outcome of the reciprocal experiment was similar, although there appeared to be an initial adjustment period before the take-over by pCJ101 containing cells. Since DS903 cells containing the parent

Figure 5.4A

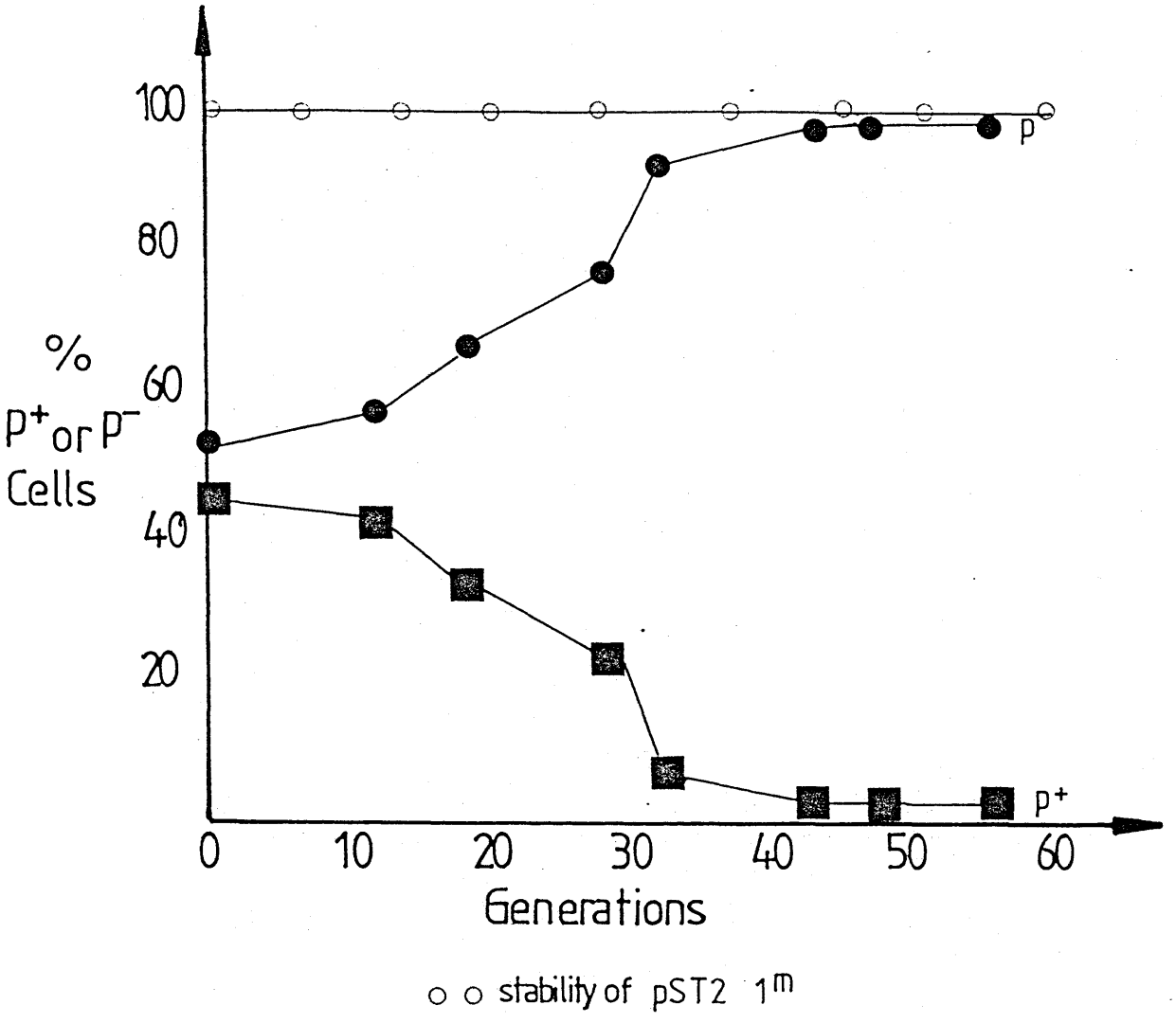


Figure 5.4A Competition Experiment Between pST2 1^{m} Containing DS903 and Plasmid Free DS903 Cells. The plasmid containing cells were resident prior to challenging with the plasmid free cells.

Figure 5.4B

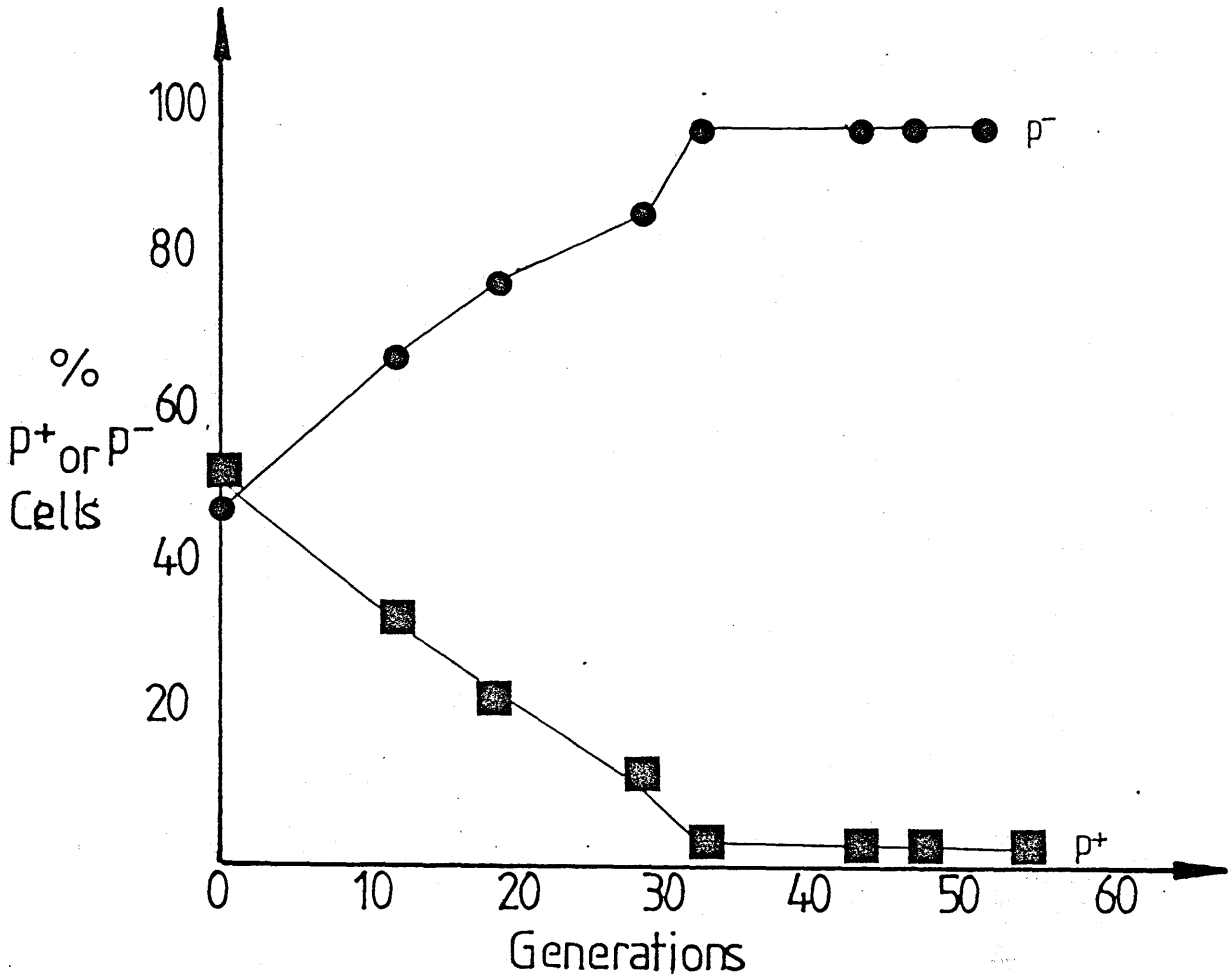


Figure 5.4B Competition Experiment Between Plasmid Free and pST2 1m Containing DS903 Cells. The plasmid free cells were resident prior to Challenging with the plasmid containing cells.

plasmid pAT153 are outcompeted by plasmid-free DS903, the above results implied that *par* may be conferring a growth advantage on the host cells.

5.4 Mixed Culture Experiments with DS903 pST2 Containing Cells and Isogenic Plasmid-Free DS903.

pCJ101 contains the pSC101 *par* region but unlike pST2, it is deleted for the 1424bp region containing the tetracycline resistance gene. In an attempt to determine whether a pAT153 derivative carrying both *par* and the Tc^r region conferred the same competitive advantage on its host as the *par*$^+$ Tc^- pCJ101 plasmid, further mixed cultures were set up and the proportion of pST2 containining cells to p^- cells monitored over 60 generations (Fig 5.4A and B). In both experiments plasmid-free DS903 cells rapidly took over the culture and after around 30-40 generations, pST2 containing cells constituted only 2% of the culture. This indicated that pST2 behaved similarly to pAT153, despite the *par* region.

CONCLUDING REMARKS

The finding that pCJ101 containing cells have a growth advantage over isogenic plasmid-free hosts contradicts the generally accepted "dogma" that the latter usually outcompete cells containing plasmids. This is attributed to the added metabolic burden conferred on bacteria especially by high copy number plasmids. Although the copy number of the three plasmids pAT153 1^m, pCJ101 1^m and pST2 1^m were not measured under chemostat growth conditions, it seems likely that any effect of the generation time (1.7 hrs) on plasmid copy number would affect all three plasmids equally. In batch culture, the copy numbers for each plasmid were similar, 23, 29 and 26 per chromosome equivalent respectively for pAT153 1^m, pCJ101 1^m and pST2 1^m. A reduction in pCJ101 copy number under chemostat conditions would reduce the growth advantage differential between plasmid-free and plasmid-containing cells, but it would not be expected to enhance the growth of p^+ cells over p^- cells.

It is unfortunate that a Tc^- deletion derivative pAT153 plasmid was not constructed during the course of this work, since this would have allowed confirmation of the possibility that *par* alone in the Tc^- pCJ101 plasmid confers a growth advantage on its host.

Assuming that the tetracycline resistance region is not involved in the growth advantage effect, these results imply that par in the pCJ101 construct may not behave as in the pST2 construct. Chapter 4 sections 4.2 and 4.3, discussed the stabilities of various multimeric forms of pCJ101 and pST2. Multimers of pCJ101 but not pST2 were stably maintained which implied that par function differed in these two plasmid constructs, however, once again this could not be directly confirmed because of the lack of a Tc^- pAT153 deletion derivative (Concluding remarks Chapter 4). It is feasible therefore that a functioning par region (in pCJ101), in addition to its role in partitioning, may increase the fitness of the host cell, although further research would be required to confirm this.

There is a precedent for specific DNA sequences dramatically altering the growth properties of E.coliK12 cells containing them. Transposon Tn5 is a composite transposon consisting of a kanamycin-neomycin resistance determinant bounded by two inverted 1.5kb IS50 sequences. The right hand element, IS50R, encodes the transposase and its inhibitor, while IS50L has an ochre mutation which results in the production of non-functional truncated polypeptides. Cells possessing the IS50R element alone have been shown to have an initial faster growth rate than their $IS50^-$ counterparts in chemostat culture (Biel and Hartl 1983; Hartl et al 1983). The enhanced growth rate effect is transient and lasts for the first 100 hours of continuous growth, thereafter, the growth rate decreases because the non-IS50 bearing cells become physiologically adapted to conditions in the chemostat (Biel and Hartl 1983). Mutational effects caused by IS50 cannot explain the enhanced growth rate, since this is independent of both the position of IS50 within the genome and also transposition of the element. Tn10 however, exhibits a similar beneficial growth rate effect, but in this case, transposition is definately involved (Chao et al 1983). Whilst the underlying mechanism of IS50 related beneficial effects remains obscure, it has been suggested that the growth rate effect may be important under conditions of extreme-nutritional shift during adaptation of organisms to new conditions. The transient selective advantage conferred by IS50 will result in the ability of cells containing it to proliferate at the expense of others, thereby maintaining IS50 in cell populations.

All chemostat studies performed in this chapter had run lengths of 102 hours (60 gen), therefore it would be interesting to determine whether

the growth advantage conferred by pCJ101 is also a transient phenomenon. Perhaps *par*, in addition to effecting accurate partition, ensures that plasmids containing it are maintained within a population of cells which would otherwise be outgrown by any plasmid-free cells which arose. An investigation into the growth properties of pSC101 *par*$^+$ and *par*$^-$ derivatives in mixed culture with plasmid-free cells, would determine whether *par* also confers a growth advantage to cells when in its natural location in pSC101. Also, since the growth advantage conferred by IS50 appears to be gene dosage dependent, it is possible that there will be a more pronounced effect in pCJ101 containing cells than in pSC101 containing cells.

CHAPTER 6

AN ANALYSIS OF THE EFFECTS OF ROM ON pUC8.

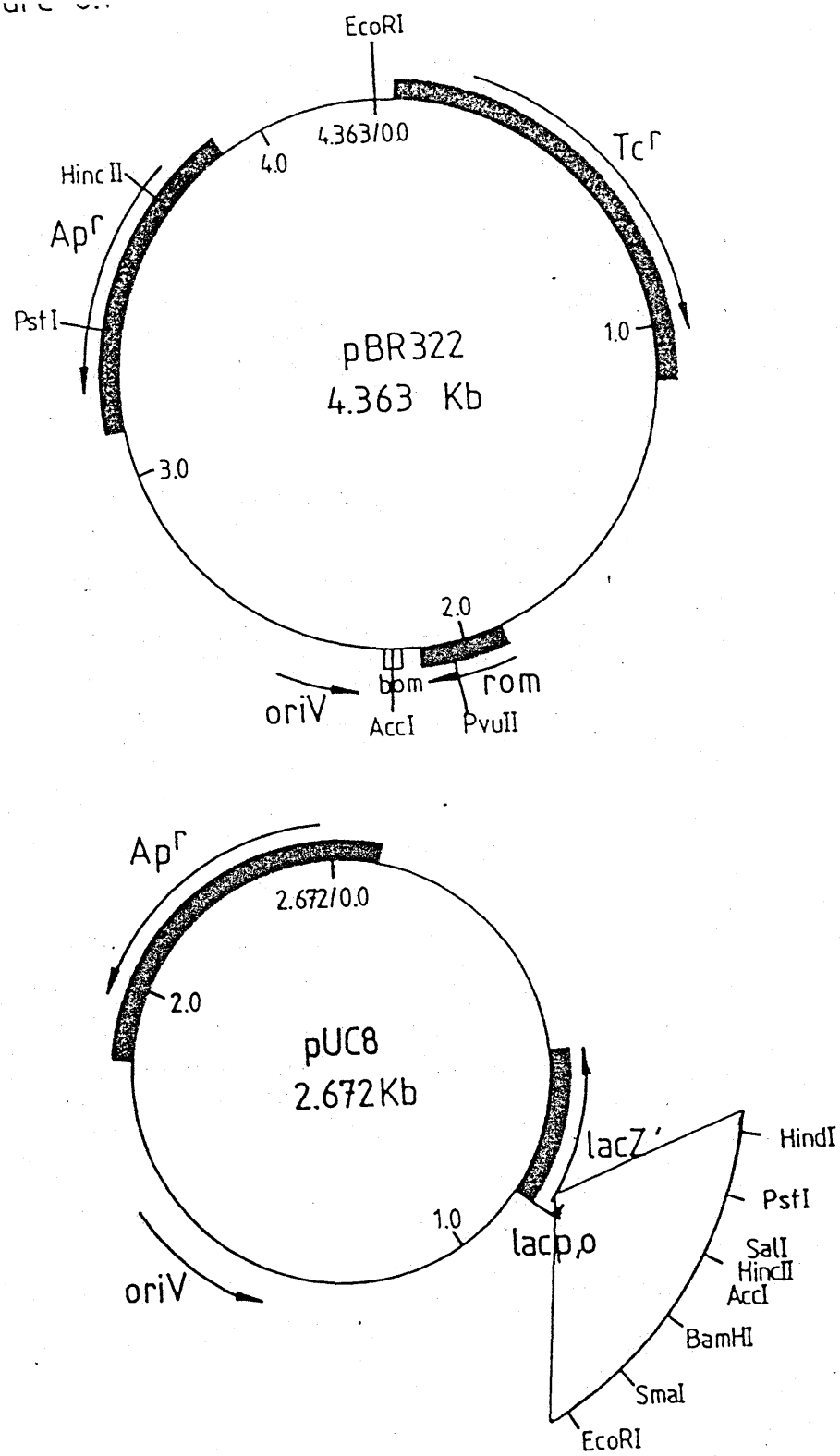


Figure 6.1 Schematic Diagram Showing the Regions of pBR322 which Remain in the pUC8 Vector. Plasmid pUC8 possesses co-ordinates 2067-4363 of the pBR322 sequence and therefore retains the bla gene and the replication origin of pBR322. It is however bom⁻ and lacks the rom gene in addition to the tetracycline resistance region. A 433bp HaeIII fragment containing the amino terminus of the lacZ gene and several unique restriction sites forms the polylinker and the basis of the cloning selection system.

INTRODUCTION

Chapter 3 dealt with the copy number and stability properties of the two related plasmid cloning vectors pAT153 and pBR322. From this work, it was concluded that when the copy number per cell is sufficiently high, the stability of these plasmids is ensured by random partitioning alone.

The recent development of the pBR322 derived cloning vectors pUC8 and pUC9 (Vieira and Messing 1982) provided an opportunity to study the stability of very high copy number plasmids. This chapter describes the construction of a *rom*$^+$ pUC8 derivative and compares its copy number and stability properties with the parental pUC8 plasmid. Various hypotheses are proposed to explain the stability of the low copy number *rom*$^+$ derivative plasmid and the instability of pUC8.

RESULTS AND DISCUSSION.

6.1 Cloning of *rom* into the pUC8 Vector in Both Orientations.

Initial observations indicated that the plasmid cloning vector pUC8 (Fig.6.1) was not stably maintained in the *rec*F strain DS903, despite its high copy number of 87 per genome equivalent (Fig 6.2 and Table 6.1). During the construction of pUC8 from the parent plasmid pBR322, the DNA region specifying the Rom protein was destroyed and consequently pUC8 is *rom*$^-$.

Twigg and Sherratt (1980) first reported that deletion of a HaeII fragment of ColE1 resulted in an increase in copy number and demonstrated that it returned to a normal level when this plasmid was co-resident with the close relative ColK. This implied that a region of ColE1 downstream of the replication origin was involved in copy number control. Subsequently, a 63 residue protein *rop* or *rom* was shown to be encoded by this region of ColE1 (Cesareni *et al* 1982; Som and Tomizawa 1983) and it has since been shown to enhance the binding of RNAI to the pre-primer RNAII several fold in addition to causing premature termination of the pre-primer RNA molecule (Tomizawa and Som 1984;

Figure 6.2

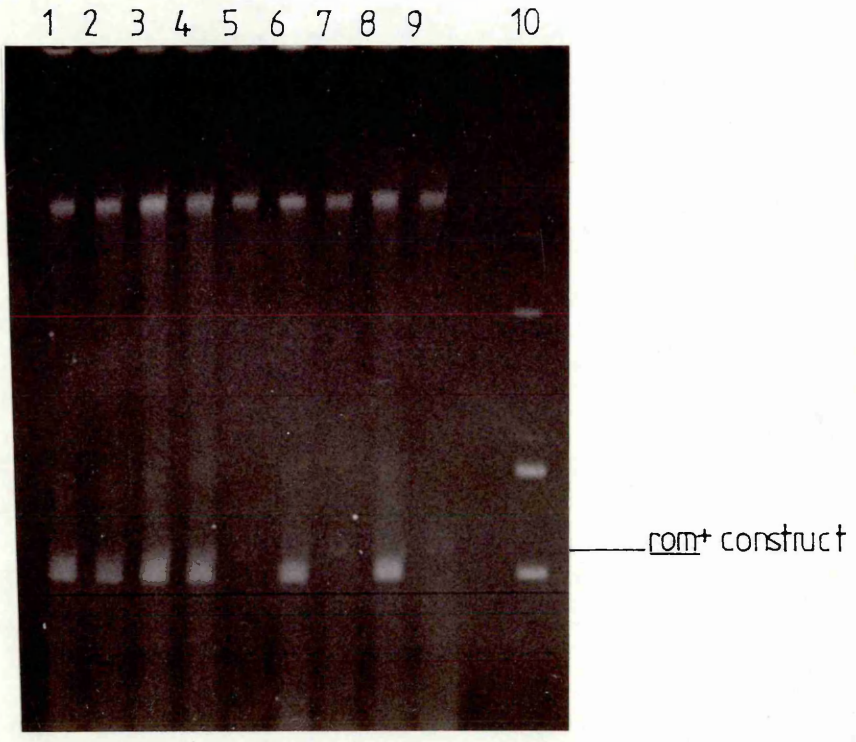


Figure 6.2 Copy Number Difference Between pUC8 and the rom+ pUC8 Constructs pCJ112 and pCJ113. 0.8% single colony agarose gel showing the clearly visible copy number difference between the rom- and rom+ pUC8 constructs.

(1) DS903 pUC8
(2) " "
(3) " "
(4) " "
(5) " pCJ112
(6) " pUC8
(7) " pCJ113
(8) " pUC8
(9) " pCJ113
(10) pUC8 marker DNA

Figure 6.3A

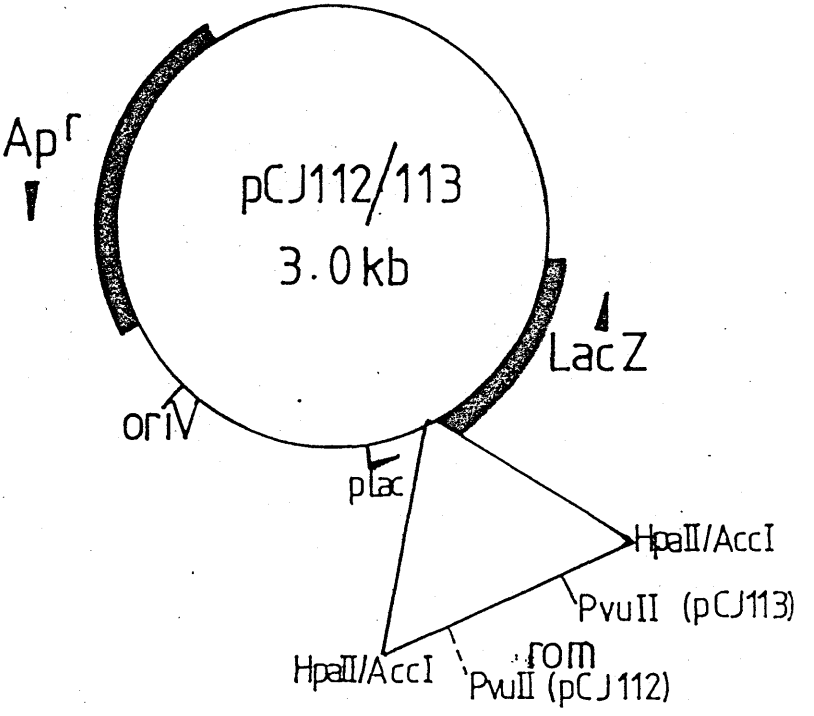


Figure 6.3A Diagram of pUC8 Constructs Containing rom. A 309bp HpaII fragment from pBR322 containing the rom gene was cloned into the AccI site in the pUC8 polylinker. Clones containing rom in both orientations were isolated. pCJ112 possesses rom in orientation 1 and pCJ113 in orientation 2.

Figure 6.3B

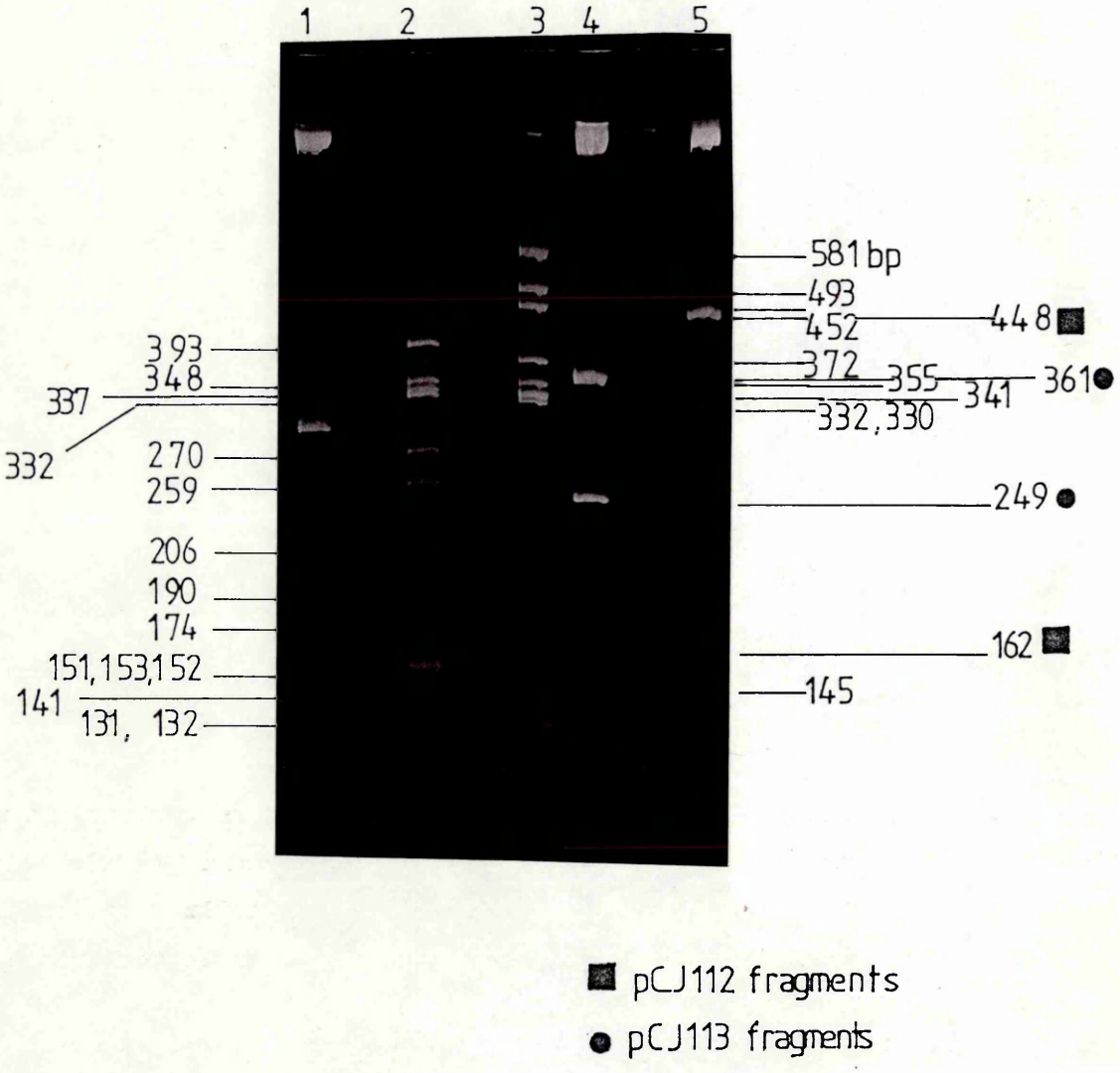


Figure 6.3B Restriction Analysis of pCJ112 and pCJ113. 5% Acrylamide gel showing the PvuII restriction profiles of pCJ112 and pCJ113. The asymmetric location of the PvuII site within the 309bp rom containing fragment allows determination of the orientation of the fragment in both clones.

(1) pUC8 PvuII
(2) pBR322 HhaI
(3) pBR322 ThaI
(4) pCJ113 PvuII
(5) pCJ112 PvuII

Lacatena et al 1984).

Since pUC8 is derived from a ColE1-like plasmid, the effect of Rom on plasmid copy number and stability could be determined by cloning a rom containing fragment into the vector. A 309bp HpaII fragment from pBR322 containing the rom gene was cloned into the AccI site in the polylinker (Fig.6.3A) and clones containing the fragment in both orientations were obtained.

Transcriptional readthrough into the origin of recombinant pBR322 derivatives was demonstrated to reduce plasmid copy number (Stueber and Bujard 1982). Since transcription of the rom gene runs towards oriV in the opposite orientation to vegetative replication in pBR322, ColE1 and ColK (Fig. 6.1) the formal possibilty existed that rom's orientation was functionally significant. The construction of pUC8 molecules possessing rom in both orientations provided an opportunity to investigate this possibility.

Plasmid pCJ112 is pUC8 with rom cloned into the poly-linker such that transcription of rom runs anti-clockwise and in plasmid pCJ113 transcription of rom runs clockwise towards the oriV region. (Fig. 6.3A;6.3B).

6.2 Rom Reduces the Copy Number of pUC8.

6.2.1 An Attempt to Estimate Copy Number Using Single Cell Resistance to Cephaloridine.

Having isolated clones containing rom in both orientations, it was necessary to determine the effect of rom on copy number. Single colony gel analysis of pUC8, pCJ112 and pCJ113 containing cells revealed that the copy number of rom$^+$ plasmids was greatly reduced in comparison to pUC8 (Fig. 6.2).

The relative copy numbers of pUC8 and pCJ112 were initially estimated by plating onto increasing concentrations of cephaloridine using the method of Uhlin et al (1977) discussed in chapter 3.

Fig. 6.4 shows the killing curves obtained for both plasmid containing strains and demonstrates that pCJ112 containing cells are less resistant to cephaloridine than pUC8 containing cells. This indicated that the copy number of the rom$^+$ plasmid was reduced in comparison to pUC8 and confirmed the single colony gel result.

Figure 6.4

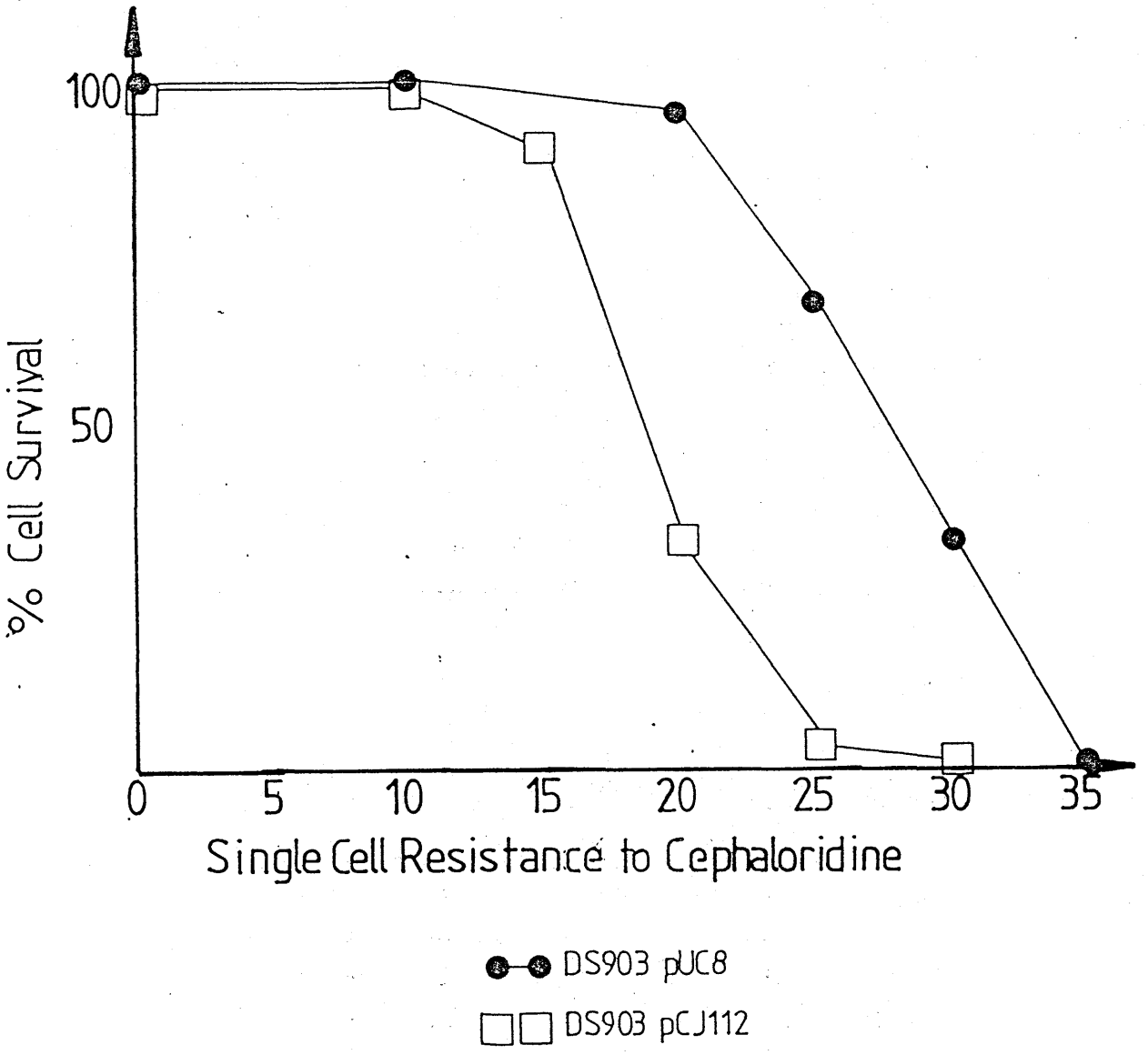


Figure 6.4 Single Cell Resistance to Cephaloridine of DS903 pUC8 and DS903 pCJ112 Containing Cells. The LD_{50} values are shown below.

DS903 pUC8 28ug/ml
DS903 pCJ112 18ug/ml

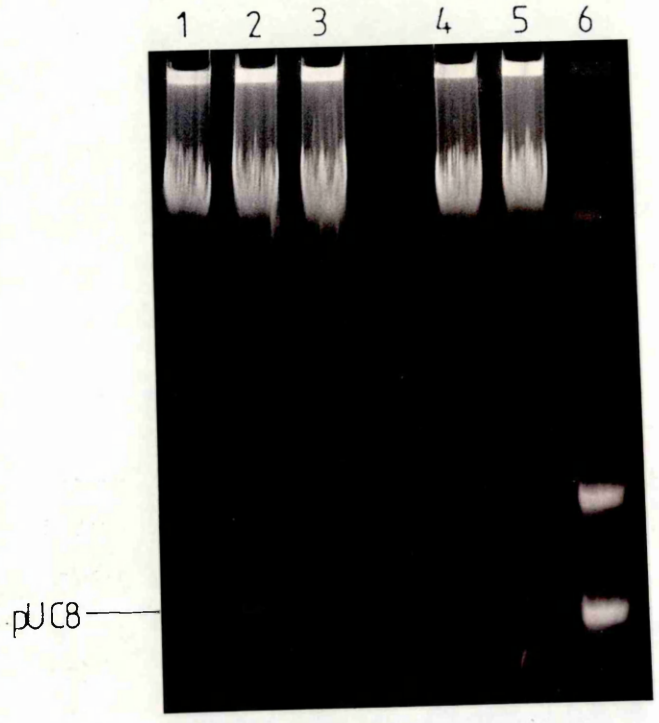
It is generally accepted that the LD_{50} point on a cell survival curve represents the minimal inhibitory concentration (M.I.C.) of a drug. By comparing LD_{50} values, relative plasmid copy numbers can be estimated. The LD_{50} values for pCJ112 and pUC8 were approximately 18 and 28ug/ml respectively. This indicates that the copy number of the rom$^+$ plasmid differs from the copy number of pUC8 by less than two fold. The single colony gel data however argues that the copy number differential is much greater.

The linear relationship between gene dosage and resistance may only hold for low copy number plasmids like R1 (1-2 copies per genome equiv.) The number of mRNA molecules estimated to be present in an E.coli cell at a given time is around 1000 (Watson 1975). It is quite conceivable therefore that a cell will not devote all of its transcription/translation machinery to the task of transcribing and translating the bla gene of very high copy number plasmids. This would result in non-linearity between gene dosage and resistance at high gene dosage levels and would explain the lack of correlation between the single cell resistance data and single colony gel analysis.

6.2.2 Copy Number Determination Using an in vivo Labelling Technique

In order to obtain quantitative data, the copy number of each plasmid was measured directly using the in vivo radio-labelling technique which was discussed fully in chapter 3. The calculated copy numbers per genome equivalent are given in Table 6.1 (see Fig.6.5). Plasmid pUC8 has a copy number of 87 per genome equivalent and pCJ112 14 per genome equivalent. As discussed in chapter 3, when calculating segregation frequencies, copy number per genome equivalent is insufficient, since information on the copy number per dividing cell is required. Both pUC8 and pCJ112 containing cells had doubling times of around 30 minutes. According to the Cooper-Helmstetter model (1968), cells with a doubling time of this order will possess approximately 3.75 genome equivalents per dividing cell. Therefore although the copy number experiments were carried out on cells representing all stages of the cell division cycle and the copy numbers obtained are average values, the copy number per dividing cell can be approximately calculated by multiplying the values expressed per genome quivalent by 3.75. The pUC8 copy number per dividing cell is then 326 and for pCJ112 53. Whatever the true copy

Figure 6.5



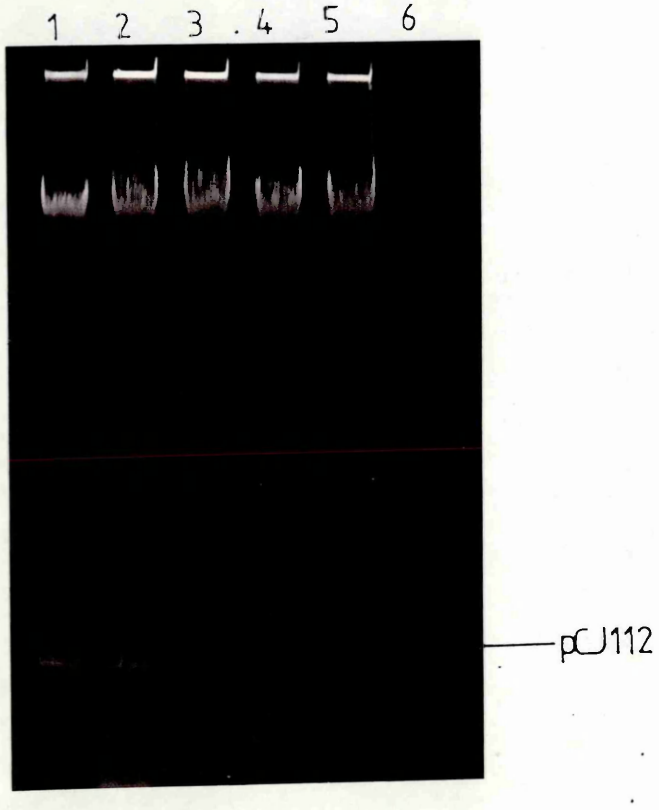


Figure 6.5 0.8% Agarose Gels Showing Total Lysates of Cells Labelled <u>in vivo</u> by ^{3}HThymidine.

Gel 1 (1) to (5) DS903 pUC8
(6) pUC8 DNA marker

Gel 2 (1) to (5) DS903 pCJ112
(6) pCJ112 DNA marker

Strain	Plasmid	Plasmid Size (kb)	Calculated Copy Number			% Standard Deviation
			(a)	(b)	(c)	
DS903	pUC8 1^m	2.7kb	87	+/- 23	(5)	26%
DS903	pCJ112 1^m	3.0kb	14	+/- 2.5	(5)	18%

Table 6.1 Calculated Copy Numbers from *in vivo* Labelling Data. Plasmid copy numbers were calculated per genome equivalent based on a figure of 3.8×10^3kb for the size of a non-replicating *E.coli* chromosome. The calculated copy numbers per genome equivalent are given in column (a), the standard deviations in column (b) and the number of independent trials in column (c). The copy number calculation was as follows:

$$\text{Copy Number} = \frac{\dfrac{\text{cpm plasmid}}{\text{cpm chromosome}} \times \text{Size of } \textit{E.coli} \text{ chromosome (kb)}}{\text{Size of plasmid (kb)}}$$

number per dividing cell, there is a 5-6 fold decrease in pUC8 copy number when rom is present in cis.

6.3. The stability of pUC8, pCJ112 and pCJ113 in DS903.

Chapter 3 discussed the importance of high copy number for stability of randomly partitioned plasmids. Since rom greatly reduced the copy number of pUC8, one might predict that the stability of the rom$^+$ plasmids pCJ112 and pCJ113 would be lower than the high copy number pUC8 plasmid.

Stability testing of pUC8, pCJ112 and pCJ113 in DS903 was performed over a period of 100 generations. Batch cultures were grown non-selectively in Davis and Mingioli salts medium and the proportion of plasmid free cells was determined every 20 generations using a starch/iodine plate assay for ampicillin resistance (Boyko and Ganschow 1982). Repeated experiments demonstrated that pUC8 was very unstable being lost from DS903 at 1-3% per generation (Fig.6.6) while for the rom$^+$ plasmids pCJ112 and pCJ113, no plasmid free segregants were detected. This suggests a segregation frequency for the latter plasmids of less than 10^{-5} per cell per generation (1000 cells for 100 generations tested).

These results demonstrated that rom in cis stabilized pCJ112 and pCJ113 despite their copy number being drastically reduced. At this stage it was unknown if the stabilizing effect was due to the reduction in plasmid copy number or to some other factor.

Since stability experiments were carried out on cells exhibiting similar doubling times to the cells in the copy number experiments, at the first approximation, the estimated copy numbers in Table 6.1 can be used to discuss the stability of these plasmids.

The rom$^+$ plasmid pCJ112 had a 5-6 fold reduced copy number with respect to pUC8 and was stably maintained. Although the plasmid containing cells were estimated to have 3.75 chromosome equivalents per dividing cell, by assuming a lower limit of 2 genome equivalents at division, the minimum copy number of pCJ112 would be 28 copies per cell. This value is more than sufficient to ensure that plasmid free cells are segregated at a frequency of less than 10^{-5} per cell per generation. Similarly assuming a lower limit of 2 genome equivalents per dividing cell for pUC8, the minimum copy number is 174 which once more is

Figure 6.6

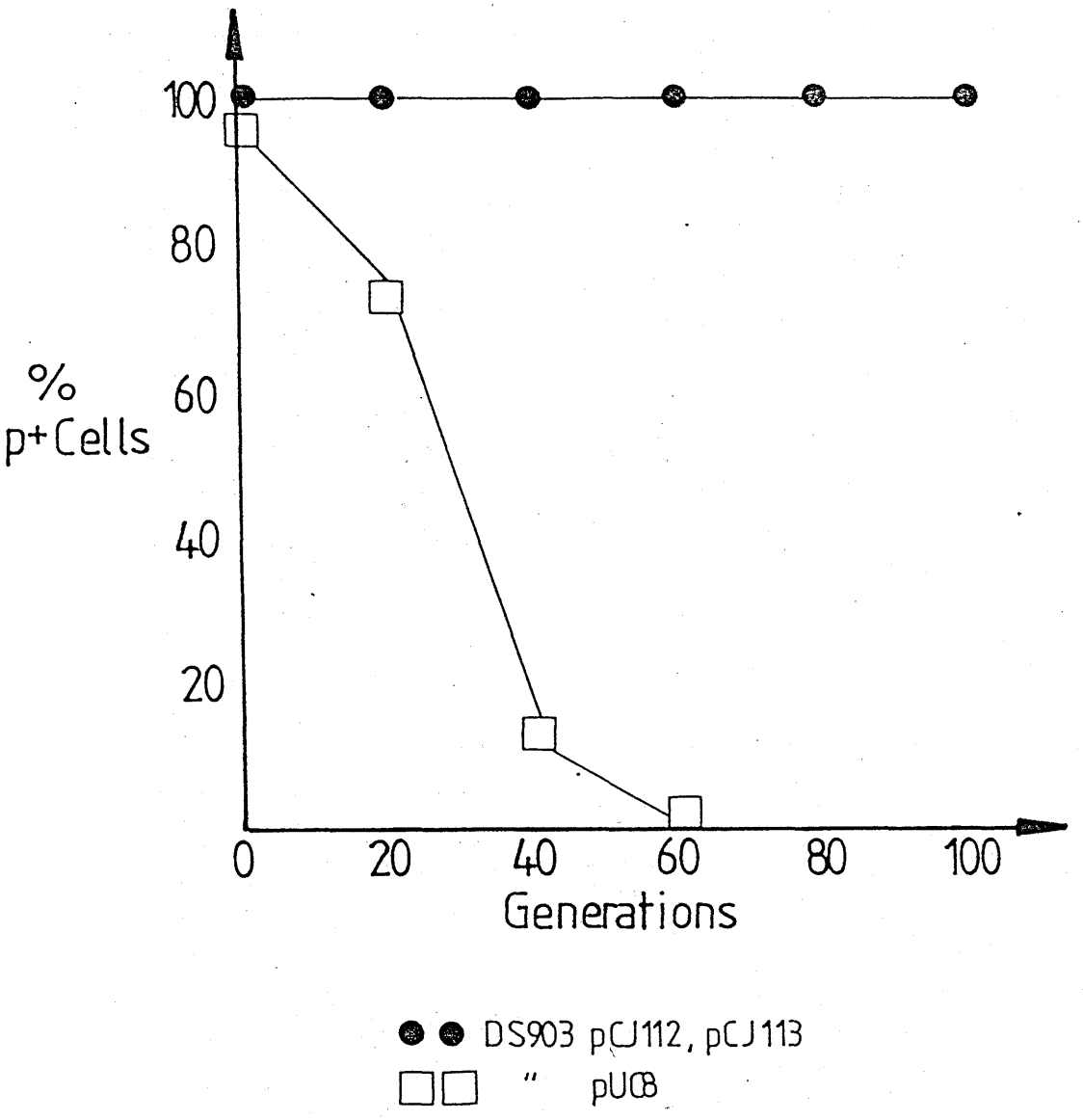


Figure 6.6 Stability of pUC8, pCJ112 and pCJ113 in DS903. The stability of pUC8 and rom$^+$ derivatives was analysed in batch culture over a period of 100 generations. The mean segregation frequencies per cell per generation are given below.

pCJ112/113	$<1 \times 10^{-5}$
pUC8	2×10^{-2}

Figure 6.7

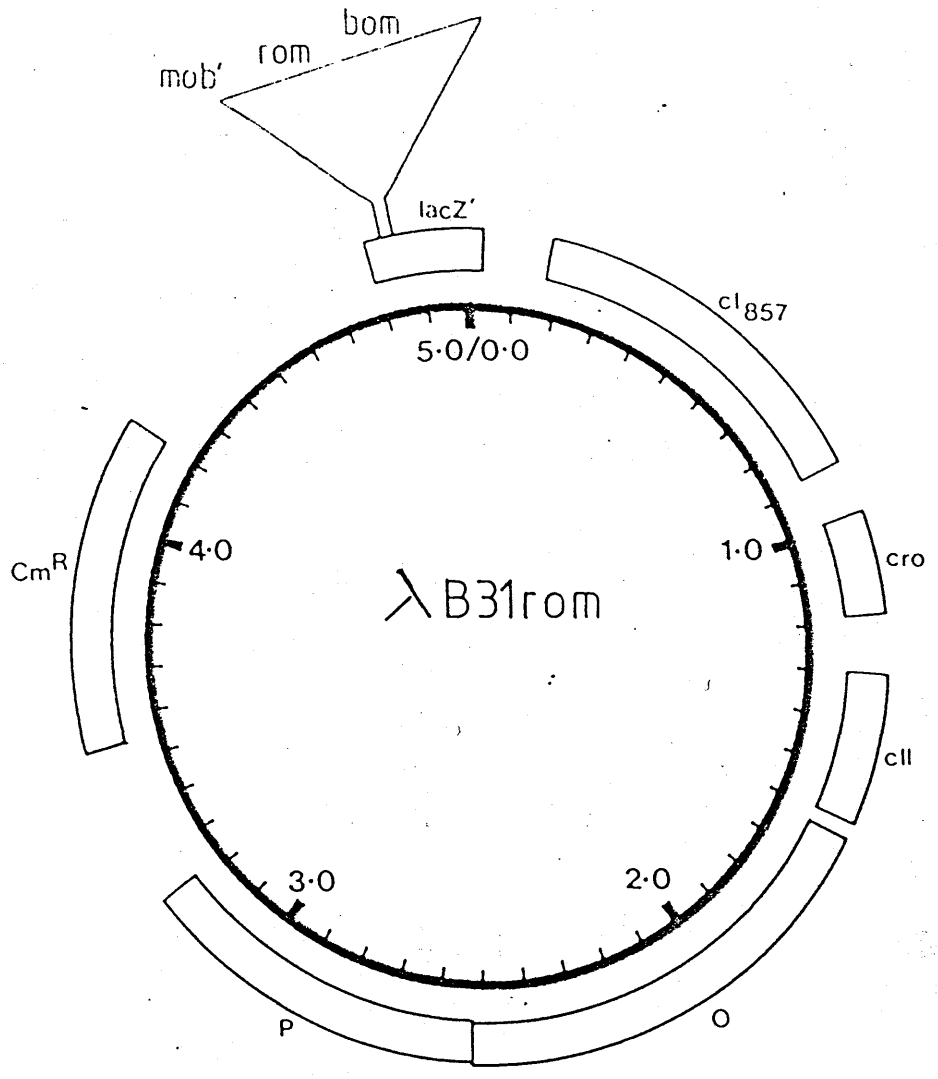


Figure 6.7 Computer Drawn Map of Lambda B31 rom+.

sufficient to ensure that plasmid free segregants occur at a frequency of less than 10^{-5} per cell per generation. Indeed, the expected segregation frequency for a copy number of 174 is $1x10^{-52}$ and the likelyhood of a plasmid free segregant arising by random segregation is therefore infinitesimal. These data indicated that factors other than copy number were affecting the stability of pUC8.

6.4. Trans-Acting Stabilization By Rom.

The rom gene encodes a trans acting repressor of plasmid copy number (Twigg and Sherratt 1980) which has been demonstrated to be a 63 residue protein (Cesareni et al 1982; Som and Tomizawa 1983; Tomizawa and Som 1984).

To determine if Rom could stabilize pUC8 in trans and to exclude the possibility that rom stabilizes in cis in a par-like manner, strains containing pUC8 and a compatible rom$^+$ plasmid were constructed. DS903 pUC8 cells were transformed by a lambda derived vector B31rom. This mini-lambda vector comprised a 3.3kb fragment of lambda containing intact cI, cro, cII, O and P genes. In addition, the vector possesses a 1.25kb fragment from pACYC184 containing the chloramphenicol resistance gene, and the polylinker region of pUC8. A 988bp Taq1 fragment from ColE1 containing bom (the region required in cis for mobilization of ColE1 by conjugal plasmids), rom and Gene1 of the mobility region was cloned into the polylinker region of the vector (C.Boyd pers. comm; Fig. 6.7).

Single colony gel analysis of the doubly transformed cells clearly showed that pUC8 copy number had decreased in the presence of B31rom, while no copy number decrease was visible in the presence of B31 alone (Fig. 6.8).

Stability experiments demonstrated that the pUC8 plasmid co-resident with the compatible B31rom vector was as stable as pCJ112 i.e. less than 10^{-5} segregants per cell per generation. In contrast, when pUC8 was co-resident with rom$^-$ B31, the instability was of the same order as that seen in cells possessing pUC8 alone (Fig. 6.9).

This data demonstrated that in addition to controlling copy number in trans , Rom could also stabilize in trans .

Figure 6.8

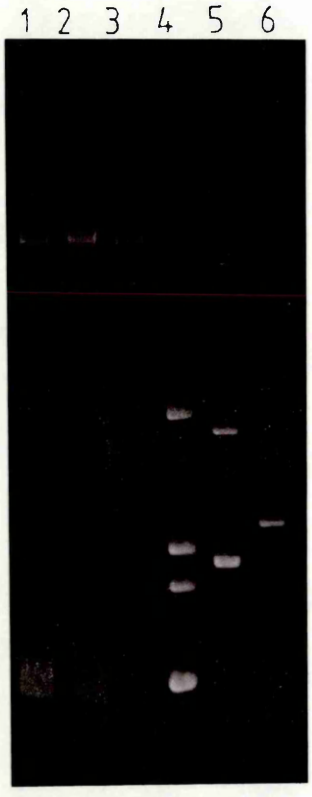


Figure 6.8 Reduction of pUC8 Copy Number *in trans* by the *rom* gene. 0.8% agarose single colony gel showing *rom* mediated reduction of pUC8 copy number in the presence of the Lambda B31*rom*$^+$ plasmid but not the Lambda B31 plasmid.

(1) DS903 pUC8
(2) DS903 pUC8 Lambda B31
(3) DS903 pUC8 Lambda B31*rom*$^+$
(4) pUC8 DNA
(5) Lambda B31 DNA
(6) Lambda B31*rom*$^+$ DNA

Figure 6.9

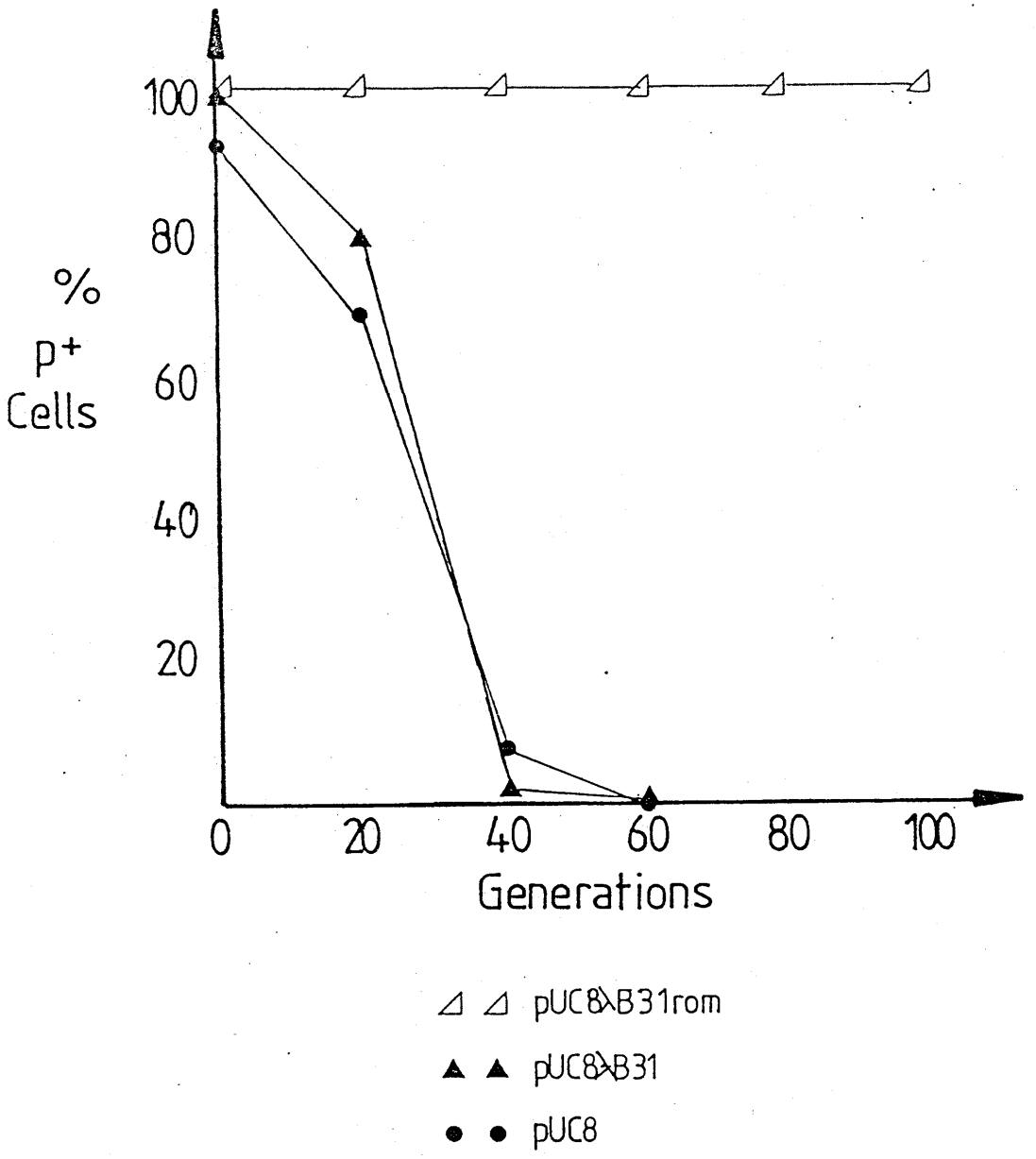


Figure 6.9 Stability of pUC8 in DS903 in Cells Doubly Transformed with either Lambda B31 or Lambda B31rom$^+$. The mean segregation frequencies for pUC8 in the presence or absence of Lambda B31 or Lambda B31rom$^+$ are given below:

pUC8 alone	2.3×10^{-2}	per cell per generation
pUC8 (B31)	2.3×10^{-2}	" " " "
pUC8 (B31rom$^+$)	$< 1.0 \times 10^{-5}$	" " " "

6.5 Possible Explanations for the Stabilizing Effect of Rom on pUC8

The stability data demonstrated that the stability of pUC8 is greatly enhanced by *rom* both *in trans* or *in cis*. The observation that Rom reduced copy number and yet enhanced plasmid stability, was contrary to the predictions of theoretical models for plasmid partitioning and to the data in chapter 3.

There are several ways in which *rom* could stabilize a plasmid.

(1) *rom* may have a "*par*-like" function which results in accurate partitioning.
(2) *rom* may have a "cer-like" function which acts to resolve multimers to monomers.
(3) *rom* could reduce the copy number variance within a cell population.
(4) *rom* could act as a "de-clumping agent" in situations where the number of segregating units has been reduced due to plasmid-plasmid aggregation.
5) The reduction in copy number by *rom* may result in a diminished growth advantage of plasmid-free cells over plasmid- containing cells so that any plasmid free cell arising will be unable to rapidly outgrow the plasmid containing cells. The severe instability of pUC8 may be a result of a large difference in growth rate between p^- and p^+ cells.

Possibilities (1) and (4) are discussed in the concluding remarks section at the end of this chapter.

6.6 Does Rom Act in a Manner Similar to ColE1 *cer* ?

A region of ColE1 designated *cer* has been implicated in plasmid stability (Summers and Sherratt 1984). *cer* has been located within a 280bp fragment of ColE1 and is a region within which site-specific recombination occurs mediated by host proteins resulting in the breakdown of plasmid multimers to monomers. Recent work (Chapter 3; Summers and Sherratt 1984) has shown that multimeric forms of plasmids are more unstable than monomers and that plasmid multimers have a reduced copy number in comparison to the monomeric form. This observation is predicted by the "origin-counting" model for copy number

Figure 6.10

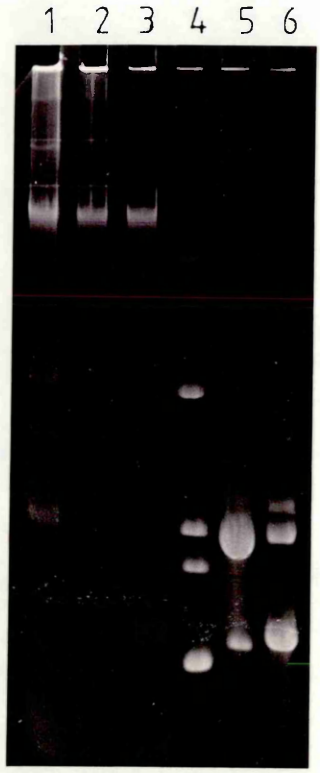


Figure 6.10 Multimer Formation in the Hyper-Recombinogenic Host JC8679. Single colony gel showing multimers of pUC8, very faint multimeric bands for the low copy number pCJ112 plasmid and predominantly monomeric plasmid in the pKS451(cer$^+$) track.

(1) JC8679 pUC8
(2) JC8679 pCJ112
(3) JC8679 pKS451
(4) pUC8 DNA
(5) pCJ112 DNA
(6) pKS451 DNA

Figure 6.11

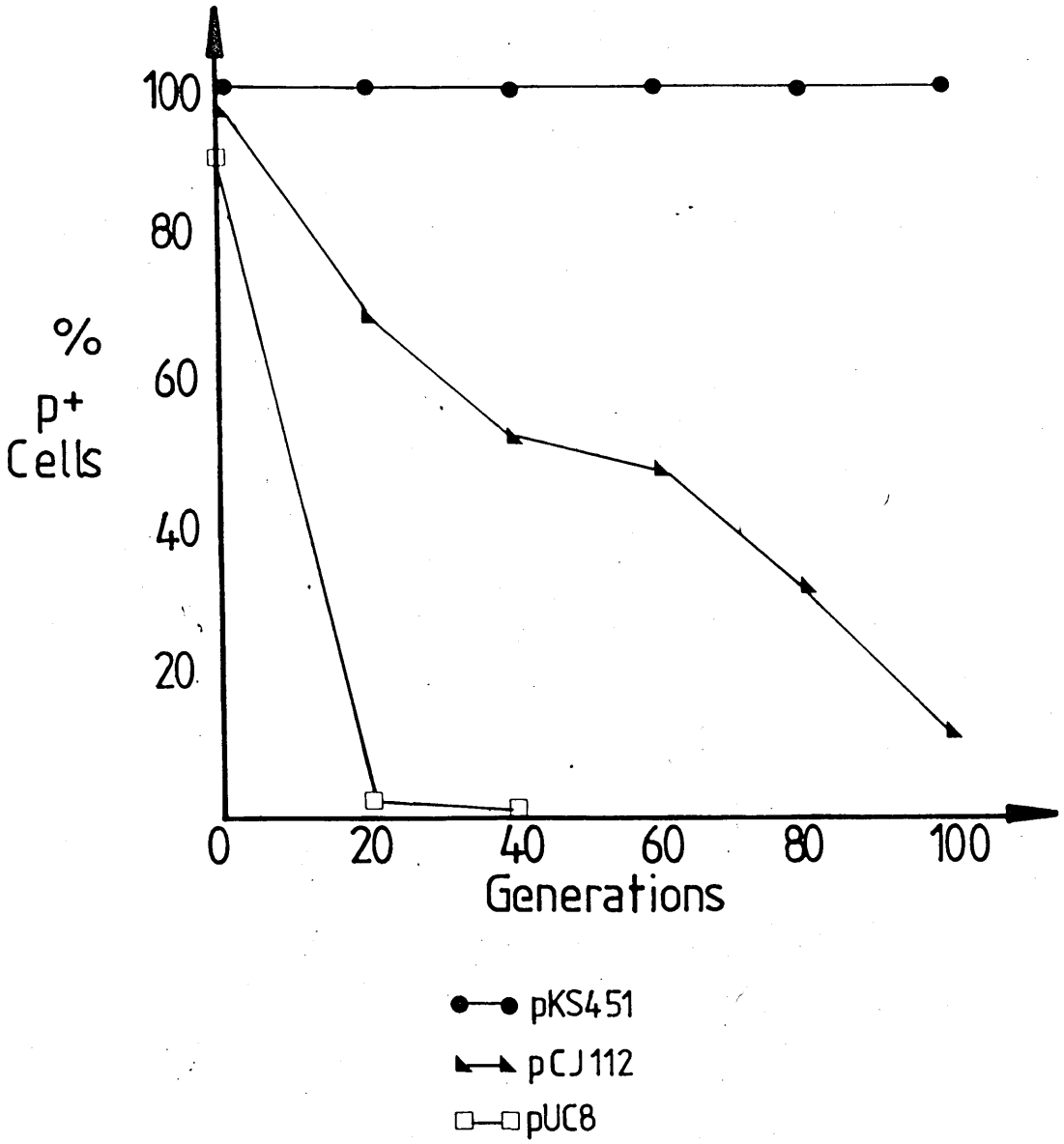


Figure 6.11 Stability of pUC8, pCJ112 and pKS451 in JC8679. The mean segregation frequencies for each plasmid are shown below:

pUC8	5×10^{-2}	per cell per generation
pCJ112	8.7×10^{-3}	" " " "
pKS451	$< 1 \times 10^{-5}$	" " " "

control (Pritchard 1978). ColE1 *cer* ensures that the number of multimeric molecules is kept to a minimum and thereby maximises the number of segregating units.

Since DS903, a *recF* strain, is deficient in plasmid recombination, any cer-like activity of *rom* would not be apparent. JC8679, an AB1157 derivative (*recBC sbcA*) is hyper-recombinogenic for plasmids (Fishel *et al* 1981). If *rom* acts like *cer*, the majority of plasmids in this strain should be monomeric.

pUC8, pCJ112 and pKS451 (pUC8*cer*) were transformed into JC8679 and single colony gel analysis revealed that pKS451 existed mainly as monomers with some higher forms visible. In contrast, pUC8 and pCJ112 existed entirely as multimers with very little evidence of monomers indicating that *rom* has no cer-like function (Fig. 6.10).

The stability data (Fig. 6.11) showed that pUC8 was highly unstable in this strain, while *rom* improved but failed to fully stabilize the vector. Since the majority of plasmid forms in JC8679 are multimers and therefore the number of segregating units is reduced, the probability of plasmid free cells occurring during random partitioning is greatly increased. This can explain the failure of *rom* to fully stabilize pCJ112 in this strain. No plasmid free segregants were observed for pKS451 after 100 generations. This implies a segregation frequency of $< 1 \times 10^{-5}$ per cell per generation. Single colony gel analysis demonstated that some multimerization had occurred but that the predominant plasmid form was monomeric. I would postulate on this basis, that the average copy number per cell was above the critical level required for stability of randomly partitioned plasmids.

6.7 Does Rom Affect the Copy Number Variance of Plasmids in a Cell Population?

Copy number variance was invoked to explain the instability of pAT153 and pBR322 multimers in chapter 3. Although the mean copy number of a plasmid may be high, a subpopulation of cells may possess few plasmid copies. Consequently, if the copy number control system fails to correct downward deviations in copy number, plasmid free segregants will appear at cell division. The copy number controlling systems of naturally occurring plasmids may ensure that variance is kept to a minimum. In contrast, the controlling elements remaining in engineered

Figure 6.12

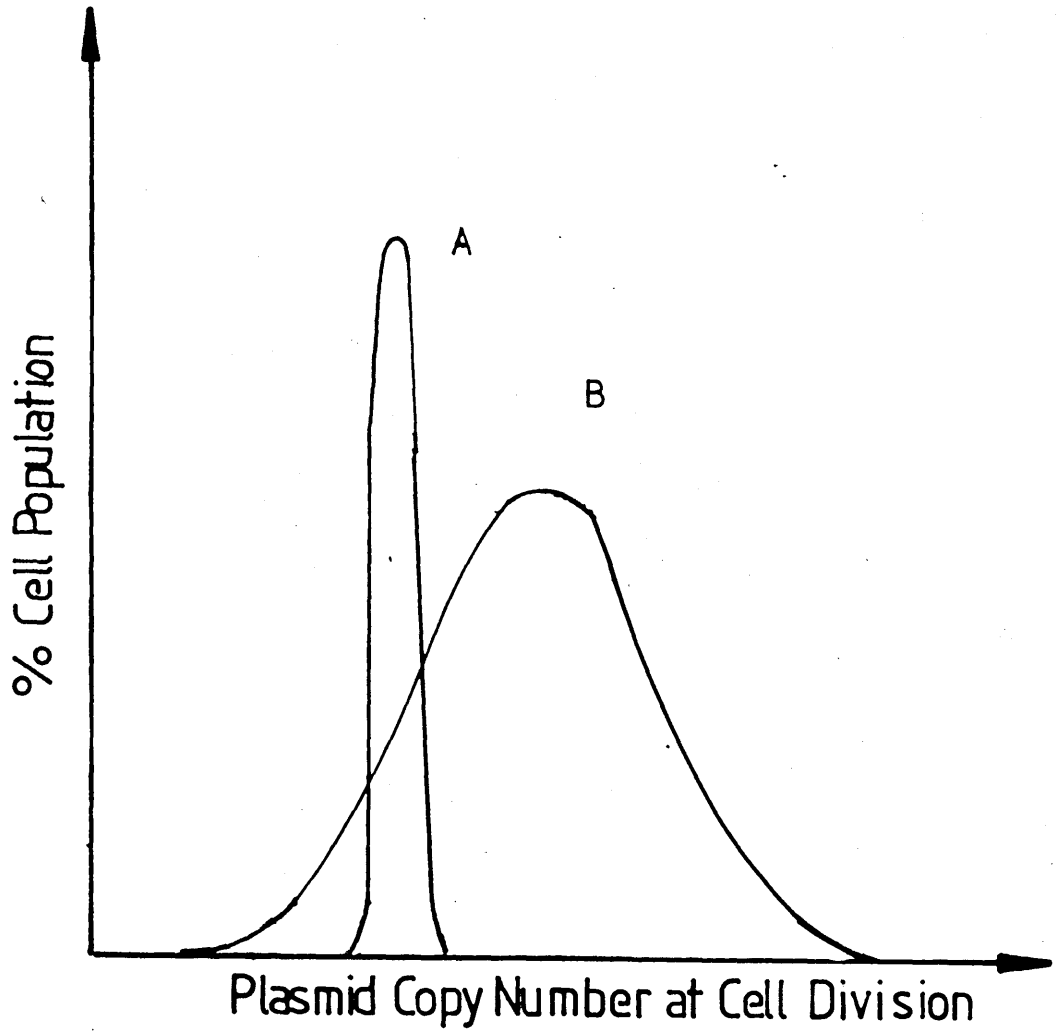


Figure 6.12 Postulated Copy Number Distributions Plasmids exhibiting (A) low and (B) high copy number variance.

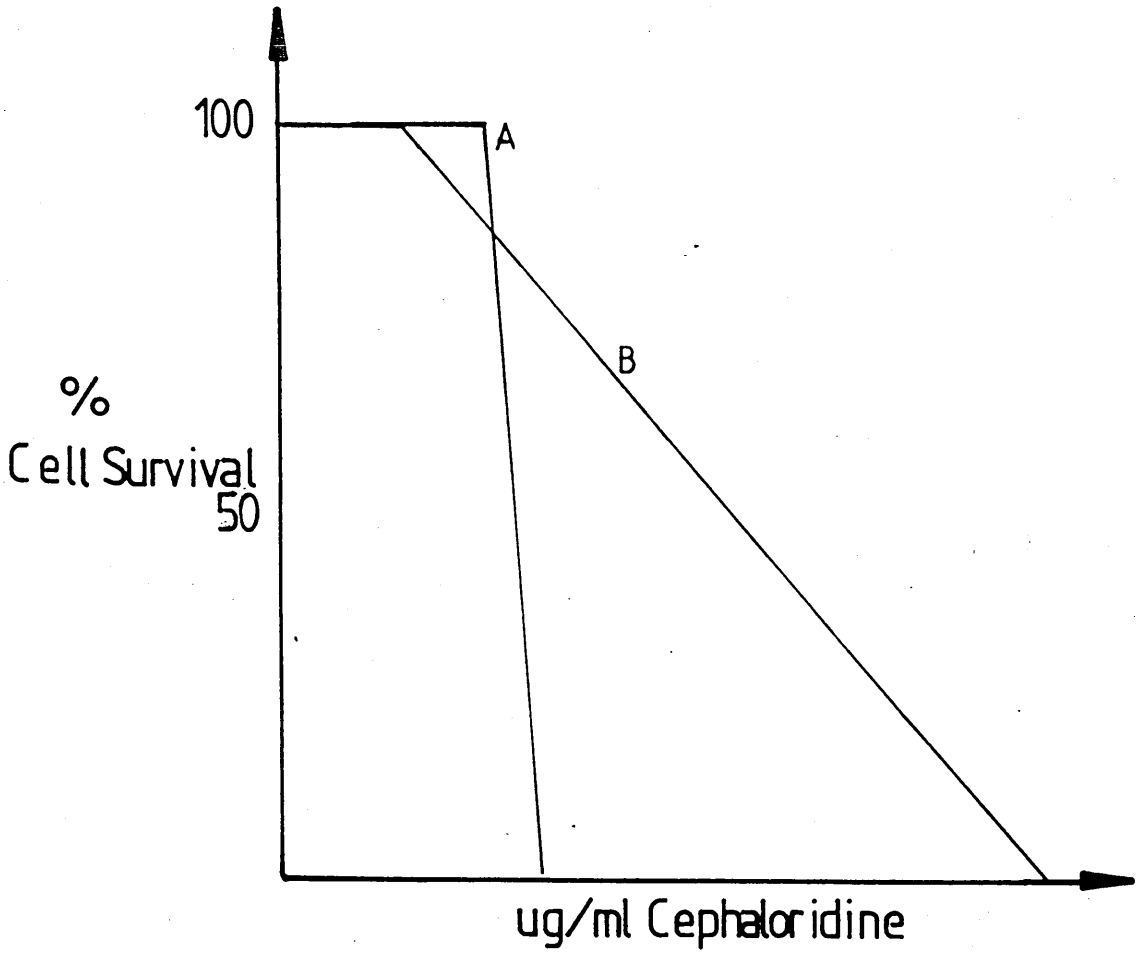


Figure 6.13 Idealised Graphs for Single Cell Resistance to Cephaloridine of Plasmids Exhibiting Low or High Copy Number Variance. A strain carrying a plasmid exhibiting low copy number variance will be resistant to a particular concentration of the drug. Above this concentration most cells will die within a very narrow drug concentration range. Cells carrying a plasmid with high copy number variance will be resistant to a much broader drug concentration range. A = low copy number variance, B = high copy number variance.

vectors such as pUC8 and pAT153 however, may be unable to effectively limit variance.

The copy number control system of ColE1 related plasmids requires not only RNA1 but also the 63 amino acid Rom protein. Recent research by Cesareni *et al* (1984) and Tomizawa and Som (1984) has demonstrated that negative regulation by *rom* requires RNAI. Since naturally occurring plasmids possess both controlling elements and many plasmid cloning vectors lack the region encoding *rom*, it seems reasonable that the copy number of these engineered vectors may not be as tightly controlled as their natural parents. This could result in copy number variance within a population of cells at cell division (Fig.6.12). Such a hypothesis could explain the instability of the high copy number pUC8 vector.

Two methods were used to try to demonstrate (a) variance in copy number in individual cells and (b) if *rom* stabilized pUC8 by decreasing variance in copy number.

6.7.1 Using Single Cell Resistance in an Attempt to Demonstrate Variance

This method measured the resistance of individual cells in a population to increasing concentrations of cephaloridine. This method should detect the resistance of individual cells because colonies arising on cephaloridine plates are due to the initial growth of a single cell surviving on a particular drug concentration. If all cells carrying a particular plasmid have similar copy numbers, there will be almost 100% cell survival until a certain drug concentration, at which point, the cells will die. In contrast, if the copy number of a plasmid varies between individual cells, then those cells with few plasmid copies will die at lower cephaloridine concentrations than cells possessing greater plasmid numbers. The idealised graphs in figures 6.13A and B depict respectively the resistance curves for plasmids exhibiting low and high copy number variance.

The only *rom* containing pUC8 derivative available at this time was AW4. This plasmid is pUC8 based with a 988bp Taq1 fragment from ColE1 which contained gene 1 of the mobility region *mob*, *bom* (the region required *in-cis* for mobilization of ColE1 by conjugal plasmids) and *rom*. It was postulated that a difference in the shapes of the killing curves of DS903 pUC8 and DS903 AW4 would reflect whether variance was

Figure 6.14

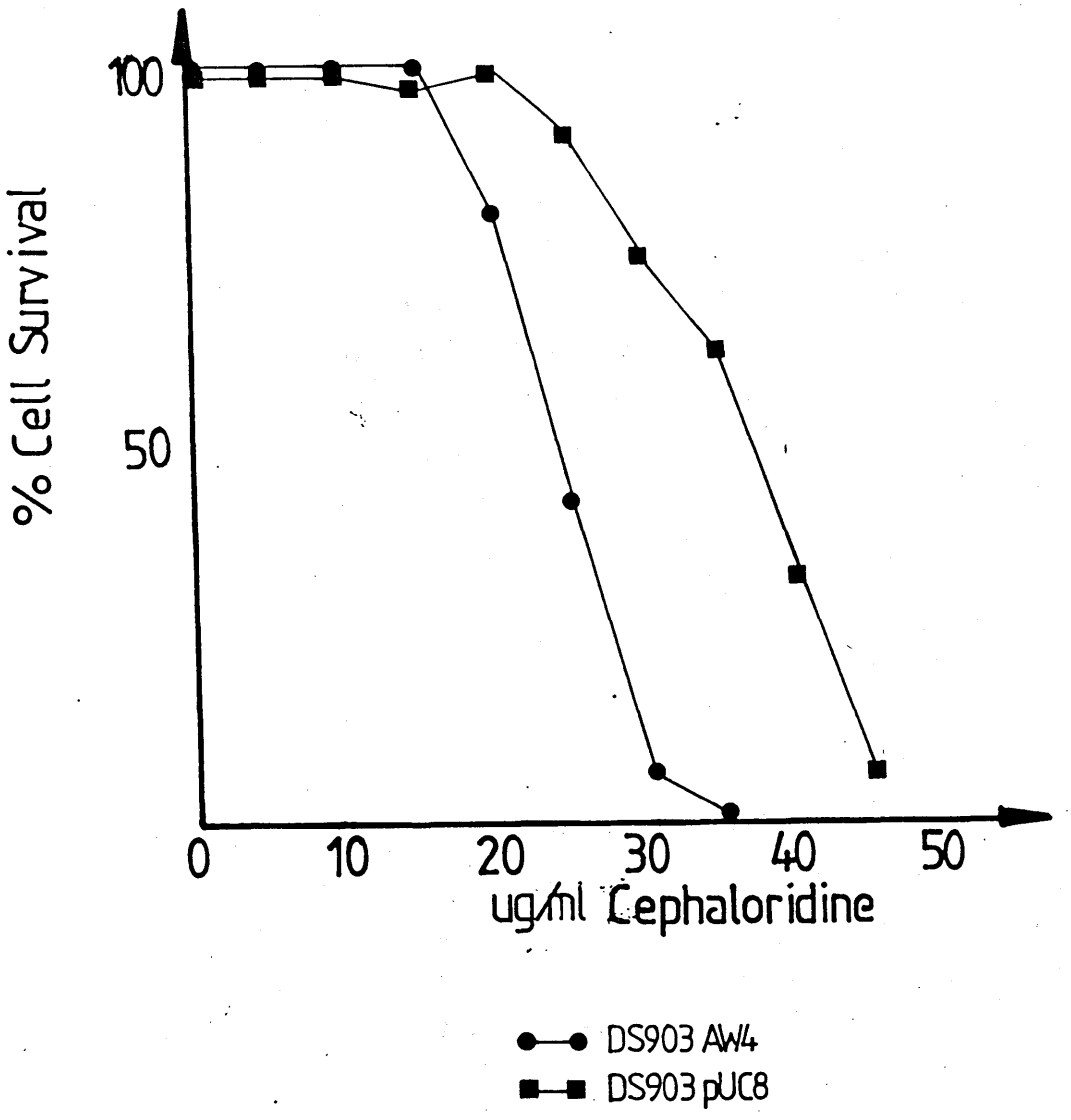


Figure 6.14 Single Cell Resistance of DS903 pUC8 and DS903 AW4. The shapes of the killing curves for both plasmids are very similar and therefore yield no information on copy number variance. The difference in copy number between pUC8 and AW4 is reflected in the LD_{50} values for each plasmid. These were as follows:

DS903 pUC8 37 ug/ml
DS903 AW4 24 ug/ml

occurring. The *rom*$^+$ plasmid was predicted to have a killing curve resembling figure 6.13A.

Several attempts at determining single-cell resistance to cephaloridine, with 4 different plates for each drug concentration, failed to produce graphs resembling the hypothetical ones in Figs.6.13A and B. Instead, the graphs reflected the difference in copy number between the two plasmid species (Fig.6.14)

From these data, it was concluded that either the system was not sensitive enough to detect variance in plasmid copy number or that variance did not exist. The inability of this technique to reflect the true copy number difference between pUC8 and pCJ112 was discussed in section 6.2. It is very probable that at high copy number levels, there will be a non-linear response between gene dosage and resistance to cephaloridine, and the likelihood of detecting copy number variance by this method was considered to be minimal. For this reason the experiment was not repeated with pCJ112 which contains the smaller *rom* containing fragment.

6.7.2 Using a *polA12*$_{ts}$ Host to Demonstrate Variance.

The second approach employed to detect variance in plasmid copy number involved a *polA12*$_{ts}$ mutant of DS903.

DNA polymerase I was the first polymerase to be discovered and was assumed to be the enzyme solely responsible for chromosome replication. The discovery of viable mutants deficient in PolI led to the conclusion that it was dispensible for *E.coli* replication and further investigation showed that DNA Polymerase III was the main replication enzyme. DNA PolI appears to be mainly concerned with repairing damaged DNA and removing RNA primers.

One mutant form of PolI is known as *polA12*. This mutant has defective polymerising ability but normal 5'→3' exonuclease activity and produces a temperature sensitive form of the enzyme at 42°C. The bacteria are viable at this temperature, but are much more sensitive to U.V. irradiation and other mutagens.

ColE1 and its derivatives have an absolute dependence on PolI for replication (Kingsbury *et al* 1970; 1973). In a *polA12*$_{ts}$ mutant at the non-permissive temperature, replication of ColE1 cannot occur. Consequently, at 42°C, continued replication and division of host cells

Strain	30°C		42°C	
	Non-Selective	Selective	Non-selective	Selective
DS903	++	-	++	-
CT001	++	-	++	-
CT004	++	+	++	-
CT005	++	+	++	-

Table 6.2 Growth of <u>pol</u>A12$_{ts}$ Strains Containing the Ap^r pUC8 or pCJ112 Plasmids at the Permissive (30°C) or Non-Permissive Temperature (42°C). Plasmid containing strains were grown selectively or non-selectively at 30 or 42°C. Lack of growth at 42°C on ampicillin containing selective plates indicated that plasmid replication in CT004 and CT005 was unable to occur due to the <u>pol</u>A12$_{ts}$ lesion in the host strain. DS903 the parent strain without the <u>pol</u>A12$_{ts}$ lesion was included as a control.

CT001 = DS903 with <u>pol</u>A12$_{ts}$ lesion
CT004 = CT001 carrying pUC8
CT005 = CT001 carrying pCJ112
++ denotes good growth
+ denotes poor growth

in the absence of plasmid replication, results in plasmid copies being diluted out. Therefore, by "digging-up" a large number of plasmid containing colonies grown on non-selective 42°C plates, resuspending and plating the cells selectively at the permissive temperature and counting the resulting colonies, it should be possible to determine the number of plasmid copies in individual cells. This would indicate whether copy number variance exists in pUC8.

It was necessary to introduce a polA12 lesion into DS903, the strain used for all previous plasmid studies. The DNA polA mutation was transduced from PO810 (polA12$_{ts}$ fad::Tn10) to DS903 using a P1$_{ts}$ Km transducing phage. PO810 was infected with P1$_{ts}$ Km at 30°C and Km^r colonies obtained. These colonies were grown up at 30°C in liquid culture and shifted to 42°C for 1-2 hours in order that the temperature sensitive P1 would lyse the cells. DS903 was infected with the chloroformed transducing lysate and the cells grown at 30°C. Tc^r(Tn10) Km^r colonies were then selected and tested for MMS (methylmethanesulfonate) sensitivity at 42°C since polA mutants are more sensitive to mutagens than wild-type strains. To determine whether the obtained MMS^s colonies were indeed polA12$_{ts}$ mutants, several isolates were transformed with the test plasmids pUC8 and pCJ112. One MMS^s isolate had a very poor transformation frequency, a common feature of DNA polA mutants (D.J.Sherratt, pers comm) and Ap^r transformants were streaked to single colonies at 30°C and 42°C. Growth occurred at 30°C both on ampicillin selective and non-selective plates. In contrast, no growth occurred on pre-warmed ampicillin selective plates at 42°C but did so on non-selective plates L-plates at 42°C (Table 6.2). This result indicated that chromosomal replication can occur at the non-permissive temperature but plasmid replication is abolished. This is in accord with the data of Kingsbury et al (1970;1973).

Having constructed a suitable polA12$_{ts}$ host, and established that plasmid replication ceased at the non-permissive temperature, it was possible to use this system to investigate plasmid copy number variance.

The polA12$_{ts}$ hosts containing pUC8 (CT004) or pCJ112 (CT005) were grown selectively in ampicillin at 30°C, the temperature which permits plasmid replication. Thereafter, cells were diluted and plated onto pre-warmed L-plates at 42°C. After overnight incubation, the resulting colonies were dug up and resuspended in phage buffer prior to plating onto ampicillin containing plates at 30°C. The number of colonies

Figure 6.15

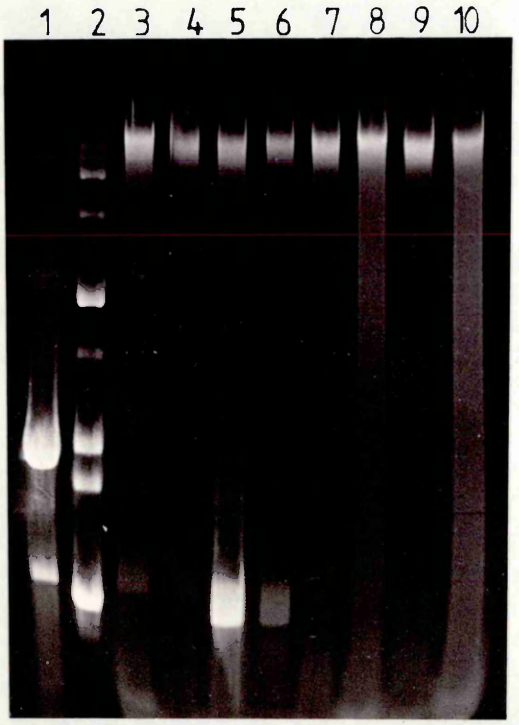


Figure 6.15 Single Colony Gel of DS903 and the polA12$_{ts}$ Strain Carrying pUC8 or pCJ112 at 30°C or 42°C. 0.8% Agarose gel showing no visible presence of either pUC8 or pCJ112 at the permissive or the non-permissive temperature in the polA12$_{ts}$ host. In contrast both plasmids are visible in the control DS903 strain at 30°C or 42°C. CT004 = DS903 polA12$_{ts}$ carrying pUC8, CT005 = DS903 polA12$_{ts}$ carrying pCJ112.

(1) pCJ112 DNA
(2) pUC8 DNA
(3) DS903 pCJ112 30°C
(4) DS903 pCJ112 42°C
(5) DS903 pUC8 30°C
(6) DS903 pUC8 42°C
(7) CT005 30°C
(8) CT005 42°C
(9) CT004 30°C
(10) CT004 42°C

appearing on these plates should equal the number of segregating units in the original cell, which gave rise to the colony "dug-up" from the L-plate at 42°C. By analysing several hundred colonies of cells containing pUC8 (CT004) and cells containing pCJ112 (CT005) it should have been possible to demonstrate variance in copy number if it existed.

There were two main problems with this system. Firstly, single colony gel analysis carried out primarily to show the absence of plasmid copies at the non-permissive temperature also revealed that the copy number of pUC8 and pCJ112 at the permissive temperature (30°C) was extremely low (Fig. 6.15). In addition, stability experiments carried out at 30°C and 42°C demonstrated the severe instability of these plasmids at both temperatures (Table 6.3). Even under selective conditions after overnight growth at 30°C only 5% of the pUC8 containing cells and 23% of the pCJ112 containing cells still retained plasmids. Given this, it was unlikely that accurate results would be derived from this *polA12*$_{ts}$ host, however, several attempts were made to try to determine the number of segregating units per cell using this system. On each occasion, 20 colonies of CT004 (*polA12*$_{ts}$ pUC8) and CT005 (*polA12*$_{ts}$ pCJ112) were dug up from L-agar plates at 42°C. The cells were resuspended in phage buffer and plated onto ampicillin plates at 30°C. As a control, cells grown non-selectively at 30°C were treated likewise without growth at 42°C. The results from these small scale experiments were rather inconclusive. Very many of the colonies grown non-selectively at 42°C yielded no Ap^r colonies at 30°C. This was not entirely surprising considering the instability of these plasmids (Table 6.3). Those giving rise to Ap^r colonies at 30°C, produced only a few and this number did not exceed 20 and no difference in copy number, judged by colony formation was observed between pUC8 and pCJ112. Control cells grown non-selectively at 30°C however, produced confluent growth on ampicillin plates at 30°C.

Because of the failure to detect copy number differences between pUC8 and pCJ112, it was concluded that this system was not sufficiently sensitive to detect copy number variance.

6.8 Does a Decrease in Plasmid Copy Number Increase the Fitness of the Plasmid Containing Cell?

There have been several reports in the literature of plasmid free

Strain	% Plasmid-free		
	0 generation	20 generations 30°C	20 generations 42°C
CT004	95%	100%	100%
CT005	77%	100%	100%

Table 6.3 Stability of pUC8 and pCJ112 in the *polA12*$_{ts}$ Host Strain. CT004 = DS903 *polA12*$_{ts}$ containing pUC8, CT005 = DS903 *polA12*$_{ts}$ containing pCJ112.

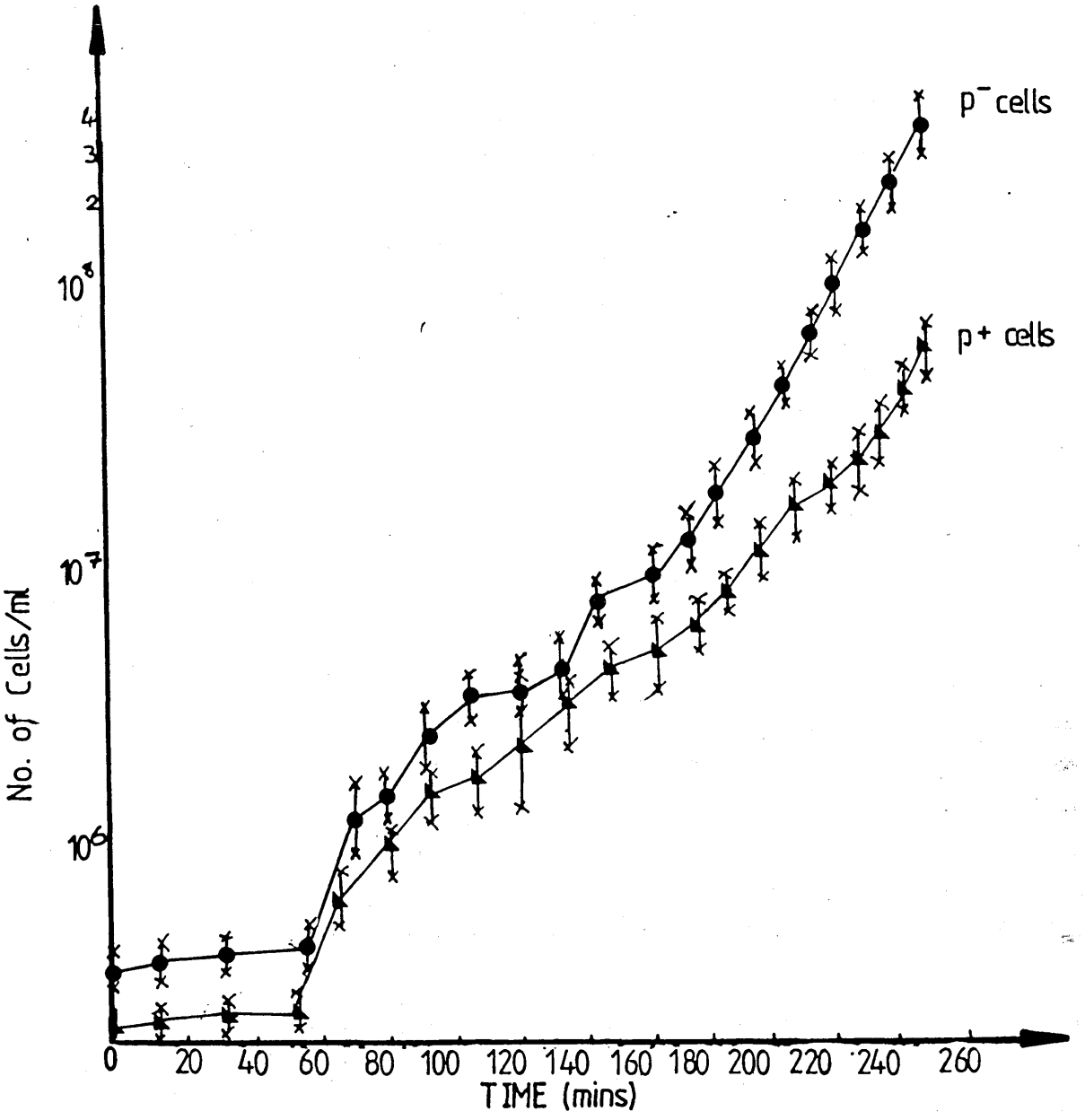


Figure 6.16A Competition Between DS903 pUC8 Containing Cells and Plasmid-Free DS903. A mixed culture was set up containing pUC8 containing cells and the plasmid-free host strain DS903. The fate of each component in the culture was followed over several hours growth by sampling frequently and determining the number of plasmid-free and plasmid containing cells at each interval. The graphs are essentially parallel until around 180 minutes of growth when they begin to diverge and the plasmid-free cells outgrow the plasmid-containing cells. The doubling times of pUC8 containing cells and plasmid-free cells were estimated to be around 23-27 minutes and 20-21 minutes respectively.

cells having a growth advantage over isogenic plasmid containing cells in mixed culture (Godwin et al 1979 ; Jones et al 1980 ; Wouters et al 1980 ; Helling et al 1980).

The maintenance cost to cells containing plasmids involves the additional replication of plasmid DNA, synthesis of RNA and protein. Under non-selective growth conditions, cells maintaining plasmids will be disadvantaged since the characteristics coded for by that plasmid are dispensible. It follows, that the higher the copy number of the plasmid, the greater the metabolic burden on the host cell. Since pUC8 has a very high copy number, it was possible that any p^- segregants arising at cell division would have a faster growth rate than pUC8 containing cells. Consequently, p^- cells would rapidly take over the culture.

Comparison between liquid culture cell densities of DS903 pUC8, DS903 pCJ112 grown selectively and DS903 after overnight growth revealed that $pUC8^+$ cells grew very poorly in comparison to pCJ112 containing cells or plasmid free cells. This observation provoked the idea that pUC8 stability experiments may give a false impression of the segregation rate of this plasmid. If competition occurs, then stability experiments will measure not only segregation frequency but rather a combination of competition between the p^- segregants and p^+ cells in addition to plasmid segregation frequency.

A mixed culture experiment was set up in an attempt to determine if $pUC8^+$ cells have a reduced "fitness" in comparison to plasmid free cells in batch culture. DS903 pUC8, DS903 pCJ112 and DS903 cells from individual overnight cultures were used to inoculate 50mls of Davis and Mingioli Minimal Medium. Two cultures were set up, one containing approximately equal proportions of DS903 pUC8 and DS903 and the other containing 50:50 DS903 pCJ112 and DS903. The cultures were shaken for 4.5hrs and sampled every 15 minutes for viable plate counts and subsequent testing for the presence or absence of plasmid via the starch/iodine B-lactamase plate test. Four duplicate plates were taken for each time point. Figure 6.16A shows the outcome of DS903 pUC8 and DS903 in mixed culture. After 180 minutes, the p^- cells in the culture grew more rapidly than the $pUC8^+$ cells since if the p^+, p^- components of the culture had equivalent growth rates, the graphs would have been parallel. The doubling times for the p^+ cells after 180 minutes were estimated to be between 23-27 min, while the p^- DS903 had a doubling

Figure 6.16B

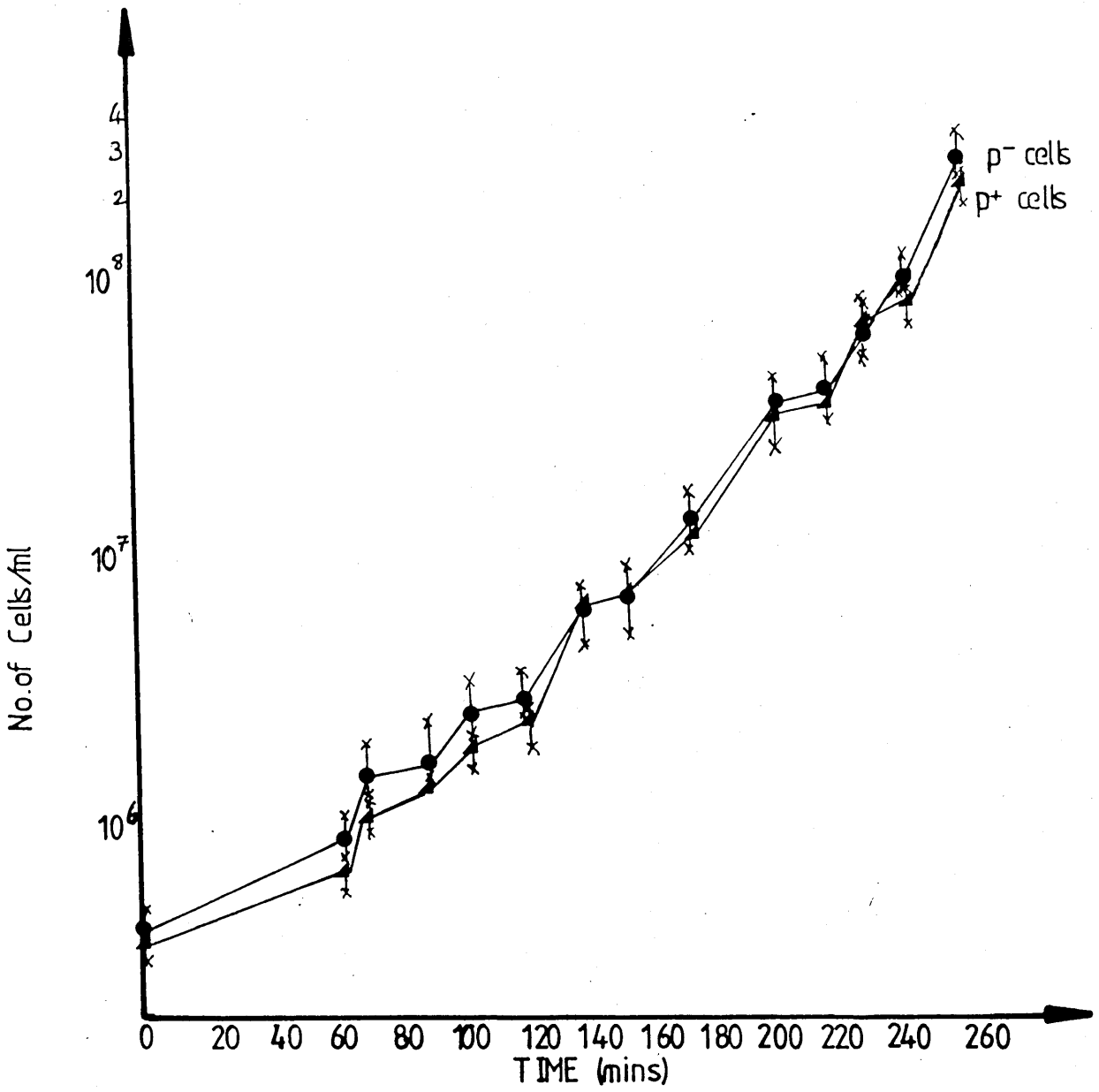


Figure 6.16B Competition Between DS903 pCJ112 Containing Cells and Plasmid-Free DS903. As figure 6.16A but pCJ112 containing cells were used instead of pUC8 containing cells. In this case the graphs remain parallel and the DS903 plasmid-free cells fail to outgrow the pCJ112 containing cells.

time of 20-21 min. This represented a difference in growth rate of between 15-28% and the plasmid free strain had the growth advantage.

Figure 6.16B shows the proportion of p^- and p^+ cells of DS903 pCJ112 in mixed culture with DS903 and demonstrated that both strains have a doubling time of around 20 minutes. Presumably, the metabolic load conferred on the host strain by pCJ112 is negligable due to the low copy number of this plasmid. Consequently, the growth of DS903 is not adversely affected.

It could be argued that some of the plasmid free cells arising during the DS903 pUC8/DS903 competition experiment were a result of segregation of the unstable pUC8 plasmid. An attempt to discover if segregation contributed significantly to the p^- component of the culture is discussed in the following section.

6.9. Using Two Differentially Marked Host Strains to Detect Segregants.

In order to determine by how much plasmid segregation contributed to the final outcome of the DS903/DS903 pUC8 competition experiment, a strain which had a different antibiotic resistance marker but was otherwise isogenic with DS903 cells was constructed. Transforming such a strain with pUC8 should allow the detection of any p^- segregants in a mixed culture experiment with the parental DS903 strain.

The strain DS903 is a streptomycin resistant strain. Such cells possess genetically altered 30S ribosomal subunits. The streptomycin resistance gene lies in a cluster of ribosomal protein genes (Bachman 1983). Spectinomycin resistant cells arise when one of the genes within this cluster (rpsE) produces a mutant protein which is insensitive to spectinomycin. Since the loci for Stp^r (rpsL) and Spc^r (rpsE) are adjacent on the E.coli map it was feasible to exchange one genetic marker for the other by generalized transduction via P1 phage.

A Spc^r Stp^s E.coli strain DS835 was infected with phage P1 and the lysate obtained was used to transfect DS903 to spectinomycin resistance. Twenty Spc^r DS903 colonies were obtained. If these were a result of transduction, the cells should have been Stp^s in addition to Spc^r due to co-transduction of these closely linked markers (both map at 72 mins. on the E.coliK12 map Bachman 1983).

Streaking out the 20 Spc^r colonies onto plates containing streptomycin and spectinomycin revealed that 19 were Stp^s Spc^r and 1 isolate was

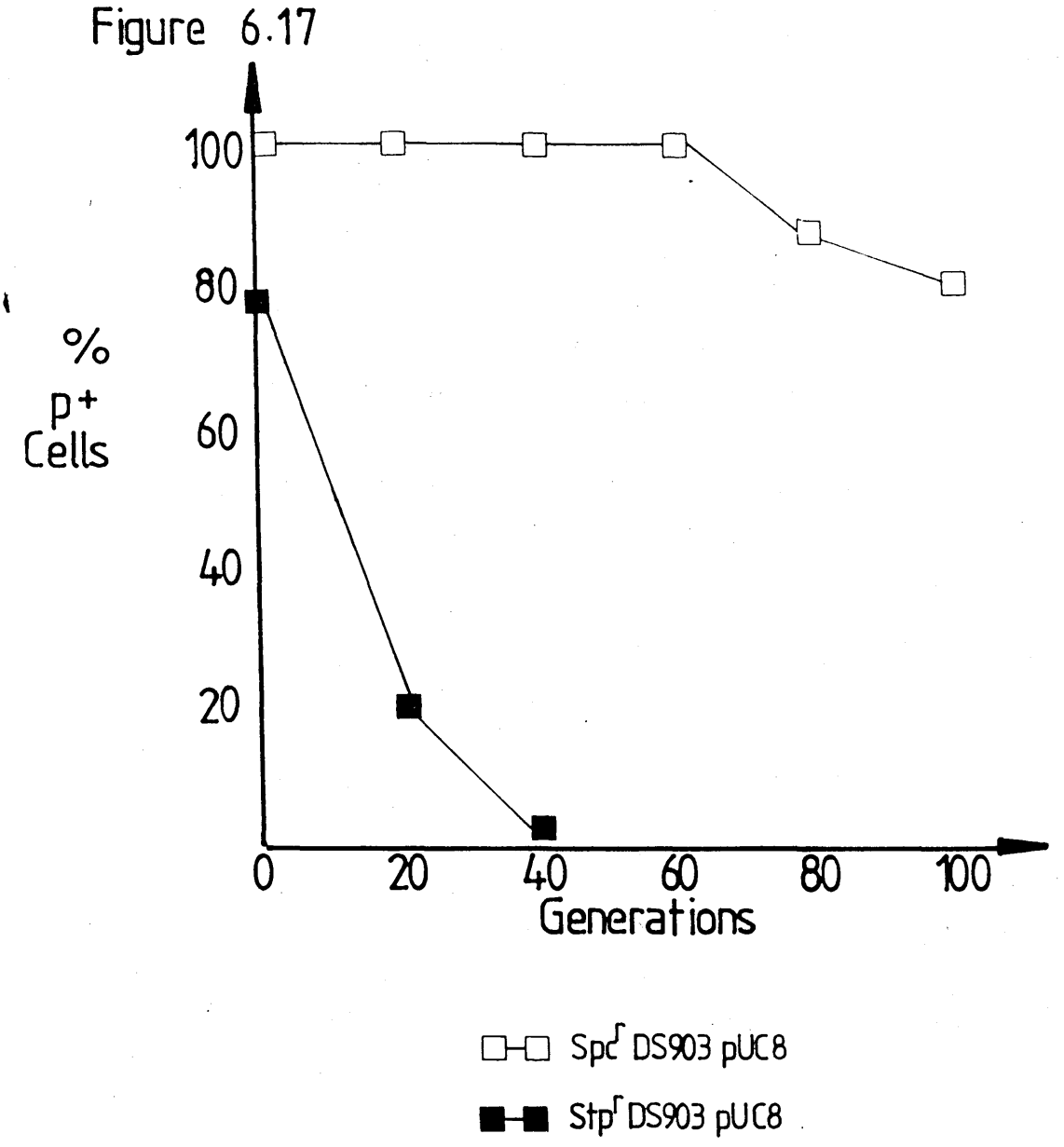


Figure 6.17 Stability of pUC8 in DS903 Stp^r or DS903 Spc^r. pUC8 is much less unstable in the Spc^r DS903 derivative than in the parental DS903 strain.

resistant to both drugs. It is probable that this doubly resistant isolate resulted from a spontaneous mutation in the rpsE gene of DS903 or from a double recombination event.

To confirm that the 19 isolates were transductants, they were screened for DS903 auxotrophic markers and were found to have a DS903 background rather than a DS835 background.

One Spc^r isolate was transformed with pUC8 DNA and Ap^r colonies obtained. By constructing mixed cultures containing 50:50 DS903 Spc^r pUC8 and DS903 Stp^r cells and vice versa, it should have been possible to detect plasmid free cells arising due to segregation during the experiment and determine the contribution to the final result of the competition experiment.

To ensure that competition experiments using this differentially marked strain would be successful, the following two criteria required to be satisfied:

(1) The Spc^r DS903 must have similar growth characteristics to its parent Stp^r DS903.
(2) pUC8 must be as unstable in the Spc^r strain as it was in the Stp^r strain.

Firstly, Spc^r DS903 grew poorly even in L-broth in comparison to Str^r DS903 and secondly, stability experiments which compared Spc^r DS903 pUC8 to Stp^r DS903 pUC8 demonstrated that pUC8 was more stably maintained in the Spc^r strain in non-selective medium (Fig. 6.17). Since this strain appears to be less "healthy" than the Stp^r strain it is possible that the copy number of pUC8 is depressed. This may reduce the competition between p^+ and p^- cells. For these reasons it was clear that Spc^r DS903 was unsuitable for such experiments. It is possible that mutations within the rpsE gene leading to spectinomycin resistance are more detrimental to the "fitness" of the bacterial cell, than are lesions in the rpsL gene (Stp^r). Alternatively, there are several ribosomal genes between the rpsE and rpsL loci which may have been mutant and would have been introduced into the DS903 cells from the DS835 strain during the co-transduction event.

Since competition experiments could not be performed with differentially marked hosts, the use of isogenic plasmid free and plasmid containing cells made it virtually impossible to distinguish

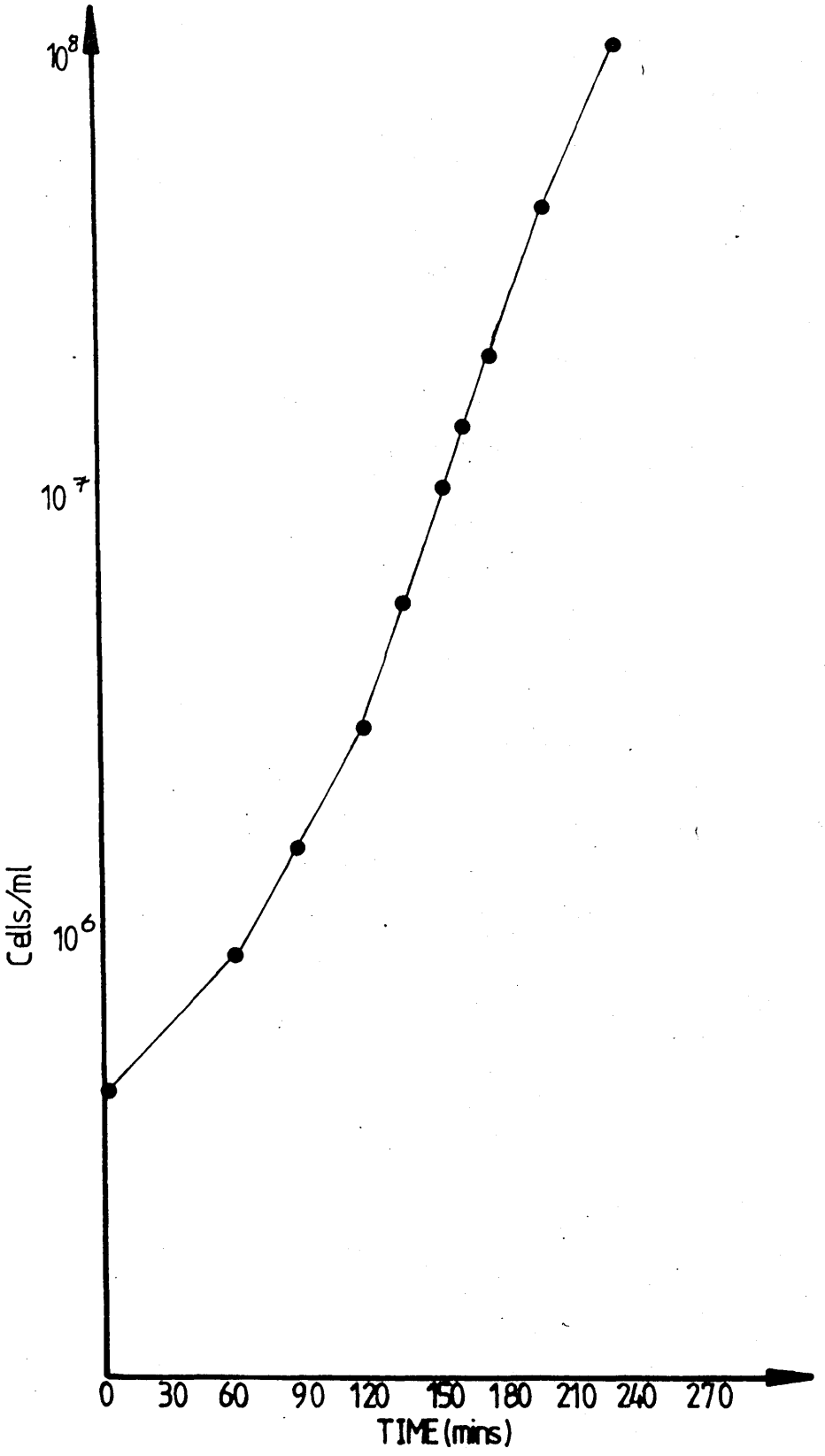


Figure 6.18 Typical DS903 Growth Curve. The estimated doubling time for DS903 was 21 minutes.

between contributions made by plasmid segregants during the experiment and the competitive effect of the p^- cells added at the start of the experiment. As a result, it was necessary to consider each contributing factor separately.

6.10 Can Segregation Alone Account for the Apparent Growth Differential in the Competition Experiments?

To determine the magnitude of contribution by p^- segregants from pUC8 containing cells which would be required to totally account for the apparent difference in growth rate between p^+ and p^- cells in competition experiments, it was necessary to make the assumption that p^- and p^+ cells have similar growth rates. By analysing a typical DS903 growth curve, it was possible to estimate by how much the addition of a particular number of p^- segregants would alter its apparent doubling time. Similarly, the increase in doubling time required for pUC8 containing cells due to segregation can also be determined.

Figure 6.18 shows a DS903 growth curve. The linear part of the graph indicates that the average doubling time is 21 mins. If in a competition experiment when there are $1x10^7$ p^- cells and $1x10^7$ p^+ cells, upon doubling to $2x10^7$ cells, the p^+ cells will yield p^- segregants with a given frequency. This will result in an alteration in the apparent doubling times of the p^+ and p^- cells. Using the standard growth equation, this alteration can be calculated.

$$N/No = e^{Kt} \qquad (1).$$

Where N = final number of cells
No = initial number of cells
K = growth rate constant = ln2/T
T = doubling time minutes
t = growth time

The doubling time from Fig.6.18 is 21 minutes and when the culture doubles then:

When $N/No = 2$, $t = T$

Using equation (1) the value of "K" can now be determined.

$$N/No = e^{KT} \qquad (1)$$

$$2 = e^{K.21} \qquad (2)$$

$$\ln 2 = K.21$$

$$0.693 = K.21$$

$$K = 0.693/21$$

$$K = \underline{0.033}$$

Since "K" has been calculated, it is possible to determine "T" the apparent generation time for p^- cells, given that a certain number of p^- segregants are produced from the p^+ component of the mixed culture. Similarly the increased generation time for p^+ cells can be determined.

For each component in a mixed culture doubling from $1x10^7$ to $2x10^7$ cells/ml and a hypothetical segregation frequency of $1x10^{-2}$, the apparent doubling time of p^- cells is calculated as follows:

$$N/No = 2-0.01$$

$$= 1.99$$

Therefore $$1.99 = e^{KT}\ p^-$$

$$\ln 1.99 = K.Tp^-$$

$$\ln 1.99 = 0.033.Tp^-$$

$$Tp^- = \ln 1.99/0.033$$

$= 0.688/0.033$

$Tp^- = \underline{\mathbf{20.85}}$ minutes.

i.e. the apparent doubling time of the p^- cells is less than 21 minutes due to the contribution of segregants from pUC8 containing cells.

The doubling time for p^+ cells is calculated as follows:

$$N/No = 2+0.01$$

$$= 2.01$$

Therefore $\quad 2.01 = e^{KTp^+}$

$$\ln 2.01 = K.Tp^+$$

$$\ln 2.01 = 0.033.Tp^+$$

$$Tp^+ = \ln 2.01/0.033$$

$$= 0.698/0.033$$

$$Tp^+ = \underline{\mathbf{21.15}}$$

i.e. The apparent doubling time for the p^+ cells is greater than 21 minutes because p^- segregants are being produced.
From the values for Tp^- and Tp^+ the relative difference in generation time can be calculated.

$$(Tp^+)/(Tp^-) = 21.15/20.85 = \underline{\mathbf{1.014}}$$

---

i.e. In the same time interval in which p^+ cells undergo 1 cell doubling, p^- cells will appear to undergo 1.014
The relative difference in cell doublings after "n" generations will be given by:

	Segregation Frequency				
Generation	10^{-2}	2 x 10^{-2}	3 x 10^{-2}	5 x 10^{-2}	10^{-1}
1	1.014	1.029	1.044	1.075	1.155
2	1.028	1.058	1.090	1.155	1.334
3	1.042	1.089	1.137	1.242	1.541
4	1.057	1.121	1.187	1.335	1.779
5	1.072	1.154	1.240	1.435	2.055
6	1.087	1.187	1.295	1.543	2.374
7	1.102	1.222	1.352	1.659	2.74
8	1.117	1.257	1.411	1.783	3.167
9	1.133	1.293	1.473	1.917	3.657
10	1.149	1.331	1.538	2.06	4.22

Table 6.4. Shows the relative difference in numbers of p^- and p^+ cells during a mixed culture experiment assuming (1) that they have equivalent growth rates and (2) that the number of p^- cells is augmented by plasmid-free segregants from the plasmid-containing component of the culture at each p^+ cell doubling of the p^+ component. The values are calculated from:

$$(Tp^+)^n/(Tp^-)^n$$

Where T = doubling time

n = generation number

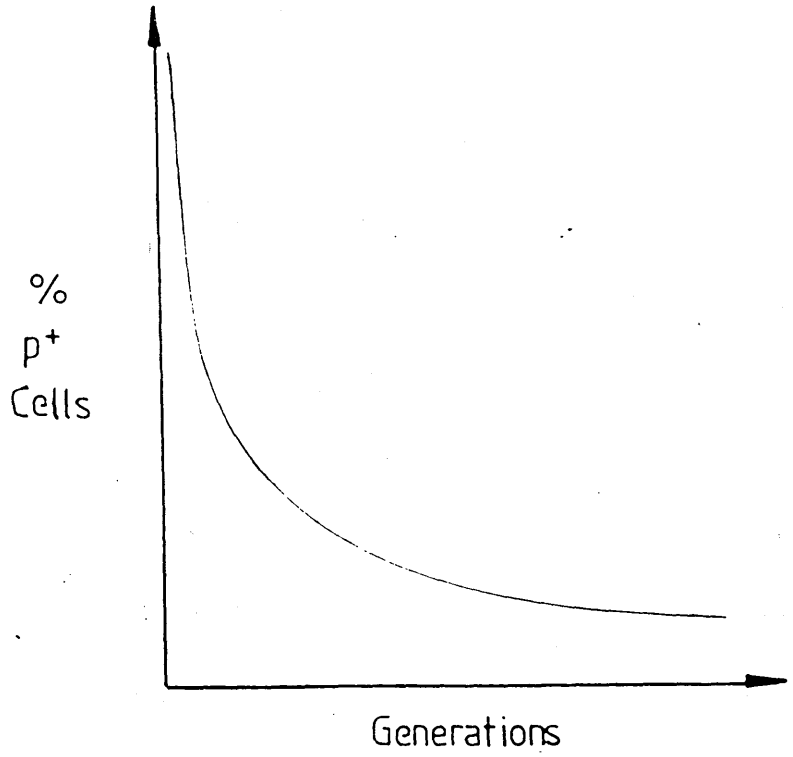


Figure 6.20

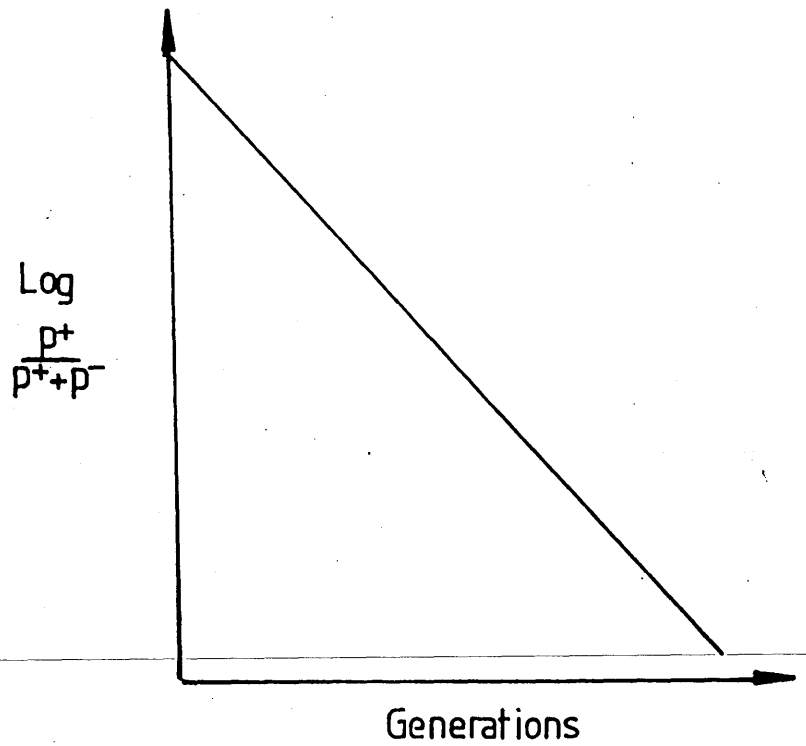


Figure 6.19 Idealised Graph of a Stability Experiment where Segregation and not Competition Dictates the Level of Plasmid Instability. If competition is not occurring between plasmid-free and plasmid-containing cells, the pattern of plasmid loss should resemble an exponential decay curve.

Figure 6.20 Log Plot of Figure 6.19.

Table 6.4 shows the relative difference in cell number of p^- and p^+ cells after several generations with a range of segregation frequencies. These data can be used to determine the segregation frequency required to explain the difference in proportion of p^+ and p^- cells at the end of the competition experiment assuming no growth difference exists between the p^- and p^+ cells (Fig.6.16A). Analysis of figure 6.16A shows that the ratio of $p^-:p^+$ at the start of the experiment was 1.6 while at the end of the experiment the ratio was 6.2. Therefore the actual final ratio after 8 p^+ doublings was 4.6. In order to attribute this result to segregation alone and not to a difference in growth rate between p^- and p^+ cells, a segregation frequency of greater than $1x10^{-1}$ per cell per generation would be required (Table 6.4). Stability experiments (Fig.6.6) show that the segregation frequency is between 1-3% per generation i.e. between $1x10^{-2}$ after 20 generations and $3x10^{-2}$ between 20 and 40 generations. Thus it seems very unlikely that segregation alone can account for the final outcome of the competitiion experiment.

Figure 6.19 is an idealised graph of the percentage of plasmid containing cells versus generations during a stability experiment, assuming that segregation is occurring and the p^- cells have no growth advantage over the p^+ cells. Figure 6.20 is a log plot of Fig.6.19. The stability graph of pUC8 in DS903 (Fig. 6.6) does not resemble the decay curve presented in Figure 6.19. Indeed, when this data was plotted onto semi-log paper it was obvious that the observed instability was not due solely to segregation (Fig.6.21). In order to explain this graph in terms of segregation only, one would require to hypothesise that segregation frequency changed as the experiment progressed. This may have been conceivable if the cells had been grown in different growth media, leading to changes in copy number during the experiment. Throughout stability testing however, large batches of minimal medium ensured that the media composition was constant.

A more likely explanation for the shape of the log plot (Fig.6.21) is that production of plasmid free segregants results in competition occurring between p^- and p^+ cells. Initially, the competition effect is not evident due to the small number of segregants being produced. After several generations, the exponential increase in the number of plasmid

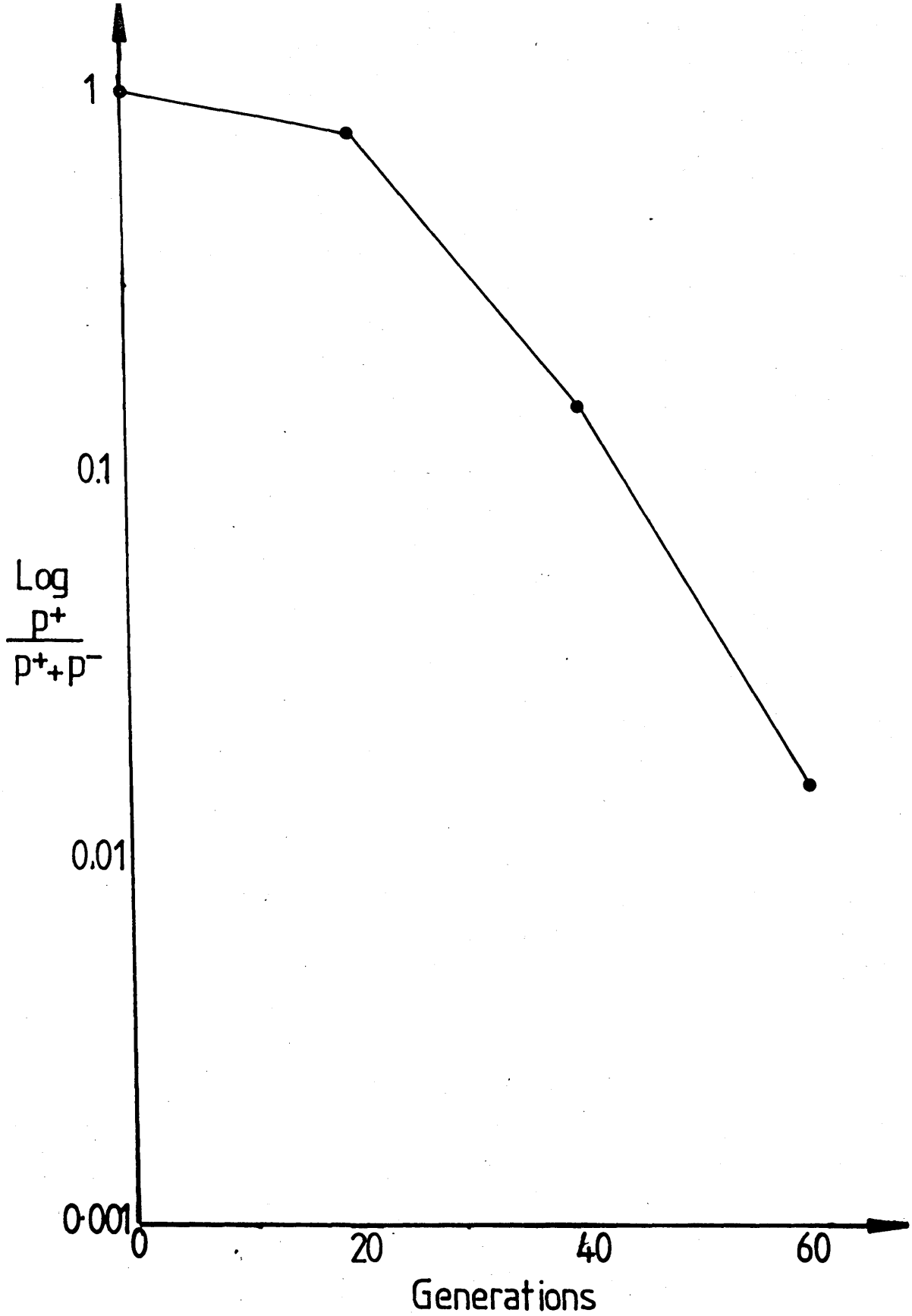


Figure 6.21 Log Plot of the Stability Data Presented in Figure 6.6. The non-linearity of the plot demonstrates that competition alone cannot account for the instability of pUC8 in DS903.

free cells is readily detected by the assay system.

6.11 Is Competition Occurring?

For competition to occur and be a contributing factor to the instability of pUC8, segregation must have already occurred. However, using a simple calculation which considers competition alone and not further segregation, it was possible to estimate the magnitude of competition required to produce the ratio of p^- to p^+ cells seen at the end of the competition experiment shown in figure 6.16A. The following assumption was made:

(i) The p^- cells arising during the competition experiment represented graphically in Fig. 6.16A were due solely to the growth of p^- cells added at the start of the experiment and not due to any segregation which may have occurred subsequently.

$$N/No = e^{kt} \quad (1)$$

Let "R" = N/No

For p^+ cells $R_1 = e^{k_1 t}$

For p^- cells $R_2 = e^{k_2 t}$

Let "Amplification Factor" = A = R2/R1

Then:

$$A = e^{k_2 t}/e^{k_1 t} = e^{t(k_2 - k_1)}$$

Where k = ln2/generation time min.
t = time of experiment min.
N = number of cells at time t
N_o = number of cells at the beginning of expt.
R = ratio N/N_o
A = "amplification factor"

By using this equation it was possible (using growth rate differences between p^- and p^+ of 10%,15%,20% and 25% over a given time) to determine the magnitude of difference between p^- and p^+ cells. During the competition experiment in figure 6.16A, the approximate generation time for p^- cells was 20 mins.

Thus: Assuming a 10% advantage p^- over p^+, over 255 minutes.

$$A = e^{t(k2-k1)}$$

$$A = e^{255(\ln 2/20 - \ln 2/22)}$$

10% Advantage	= 2.4
15% Advantage	= 3.4
20% Advantage	= 4.9
25% Advantage	= 5.9

Analysis of figure 6.16A shows that the ratio of p^-:p^+ cells at the start of the experiment was 1.6 and 6.2 at the end. Therefore, the actual final ratio was 4.6. This suggests that p^- cells have a 15-20% growth advantage over the p^+ cells, which is in good agreement with generation time comparisons of p^-,p^+ on the linear part of the graphs shown in Figure 6.16A.

The calculations in the "Segregation Only" section above, suggested that a segregation frequency of greater than 10^{-1} per cell per generation would have been required to explain the difference in the p^-:p^+ ratio at the end of the competition experiment between DS903 and DS903 pUC8. The steepest part of the pUC8 stability graph (Fig.6.6) indicated a segregation frequency of 3×10^{-2} per generation which is not sufficiently large to explain the competition data. Indeed, this value is most probably an over-estimate, since competition appears to occur and will contribute to the outcome of pUC8 stability experiments.

Figure 6.22

```
                5903       5913       5923       5933       5943       5953
COLE1      :GGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTA
            ************************************************************
PBR322     :GGACAGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTA
                2985       2995       3005       3015       3025       3035

                5963       5973       5983       5993       6003       6013
COLE1      :GCTCTTGATCCGGCAAACAAACCACCGTTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC
            *************************** ********************************
PBR322     :GCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGC
                3045       3055       3065       3075       3085       3095
```

The sequence of part of the RNAI and RNAII coding regions of ColE1 and pBR322 are shown above. The ColE1 coordinates refer to the sense strand for the rom gene numbered from the unique EcoRI site. The pBR322 coordinates are as given by Sutcliffe (1978). The loss of sequence homology at position 5981 ColE1, 3063 pBR322 results from the inc9 mutation in the latter sequence.

CONCLUDING REMARKS:

(i) Why is Rom Mediated Copy Number Reduction Less for pAT153 and pBR322 than pUC8 and pCJ112

Since pUC8 and pBR322 share the same oriV sequences, it is difficult to rationalise why Rom has a greater effect on pUC8 copy number than on pBR322. A 5-6 fold reduction in copy number was observed upon cloning a rom containing fragment from pBR322 into the plasmid cloning vector pUC8 while only a 1-3 fold reduction has been detected between pAT153 (a rom$^-$ pBR322 derivative) and pBR322 (Twigg and Sherratt 1980). Also this work (Chapt.3) has demonstrated a 1.2 fold reduction in DS903. Similar to the pUC8 data, rom$^-$ deletion derivatives of ColE1, the naturally occurring plasmid, result in a 5-7 fold increase in copy number (Twigg and Sherratt 1980).

Analysis of the DNA sequences of ColE1 and pBR322 in the region encoding RNAI and RNAII shows a T-C transition in pBR322 at position 3062 (Sutcliffe 1978) with respect to the ColE1 sequence (Fig.6.22). This sequence difference may account in part for the efficiency of Rom action in this vector.

ColE1 copy number mutant plasmids exhibiting altered incompatibility properties were analysed by Tomizawa and Itoh (1981). Sequencing data revealed that each plasmid possessed a point mutation in or near to the centre of one of the three palindromes in the DNA region specifying RNAI and RNAII. One mutant inc9, was due to a T-C transition. This is identical to the 1bp difference observed between pBR322 and ColE1 in the RNAI/RNAII region. Further studies, involving the interaction rates of various combinations of mutant and wild-type RNAI and RNAII species have shown that the binding rate of inc9 RNAI to inc9 RNAII is approximately half that observed for the "wild-type" species (Tomizawa and Som 1984). Also, the enhancement of *in vitro* binding in the presence of Rom is lower for the inc9 species than for the wild-type species. These data correlated well with *in vivo* observations on the copy number of rom$^+$ and rom$^-$ ColE1 derivative plasmids having either wild-type or inc9 RNAI/RNAII species. The copy number of the wild-type plasmid was 1.3 fold lower than the inc9 mutant, indicating that the inhibitory effect on primer formation by inc9 RNAI, is less efficient than the inhibition caused by wild-type RNAI. In addition, the decrease in copy number

caused by Rom on the wild-type plasmid was greater (3 fold) than the decrease observed for the inc9 rom$^+$ mutant plasmid (2 fold) (Tomizawa and Som 1984).

The relative insensitivity of the pBR322 replication control system to inhibition was invoked to explain the incompatibility properties exhibited by pMB1 derived plasmids (Hashimoto-Gotoh and Timmis 1981). Incompatibility tests demonstrated that pBR322 exerted a very strong form of exclusion on ColE1 derived plasmids. Since ColE1 derivatives were unable to replicate in cells carrying established pBR322 plasmids, it was suggested that ColE1 was more succeptible to replication inhibition than pMB1 derived plasmids.

The Tomizawa data suggest that the RNAI/RNAII copy number control system of pBR322 is not as effective as ColE1. Moreover, Rom mediated action on pBR322 copy number is also less effective as shown by the small copy number difference between the rom$^+$ pBR322 plasmid and the rom$^-$ deletion derivative pAT153 (Twigg and Sherratt 1980: this work Chapter 3) while, ColE1 rom$^+$/rom$^-$ plasmids exhibit a 5-7 fold copy number difference (Twigg and Sherratt 1980).

Since the Tomizawa results with ColE1 derived plasmids demonstrated only a small copy number difference between the wild-type rom$^+$ plasmid and the inc9 rom$^+$ plasmid, it is probable that other factors are affecting the copy number controlling system of pBR322.

The transcriptional environment surrounding the replication origin may influence how effectively Rom can reduce plasmid copy number. Both pBR322 and pAT153 possess a strong promoter to the right of the unique HindIII site initiating transcription in a leftwards direction towards the B-lactamase gene (Fig.6.1) which is 1.5 x as strong as the promoter for Tc^r which initiates in a rightward direction (Stueber and Bujard 1981). The leftward initiating promoter often referred to as the "anti-tet" promoter, is part of the tetracycline resistance region derived from pSC101 during construction of the pBR322 vector. In pSC101 this promoter arrangement forms part of a more complex Tc^r operon involving control of expression of tetracycline resistance. The Stueber-Bujard data strongly suggested that transcription from the "anti-tet" promoter of pBR322 proceeds into the bla gene and contributes to its expression. This was confirmed by Von Gabain et al (1983).

Since the primer promoter region of oriV in pBR322 is adjacent to the end of the bla gene, it is feasible that transcriptional readthrough

from "anti-tet" and bla promoters results in an increased rate of pre-primer RNA formation. This may interfere with the RNAI/RNAII interaction and could account for the less effective decrease in copy number by Rom in pAT153 and pBR322 compared with pUC8 and pCJ112.

By mutagenising the replication region of CloDF13, which is similar to that of ColE1, a conditional lethal, temperature sensitive copy number mutant $cop1_{ts}$, was isolated (Veltkamp et al 1981). Sequence analysis revealed that this mutation lay in a region upstream of oriV known to be involved in transcription termination. At the non-permissive temperature, transcription from Cloacin reads through the inactivated terminator into the replication origin and the increased frequency of initiation results in cell death. Therefore, ColE1- like naturally occurring plasmids possess a transcription terminator upstream of the replication origin to prevent or minimise transcriptional readthrough into this important region and perhaps this accounts for the efficient depression of ColE1 copy number by Rom. Analysis of the corresponding region of pBR322 however, revealed the presence of this terminator here also.

Von Gabain et al (1983) located three terminators at the end of the bla gene in pBR322. One of these is the natural bla terminator from Tn3, while the remaining two result from the pMB1 part of the molecule. The indigenous Tn3 terminator is relatively "leaky" since more than half of the transcripts from bla proceed beyond it. Close analysis of their data allowed the approximate position of the terminators to be located between co-ordinates 3250 and 3000 of pBR322 (Sutcliffe 1978). Z-Term, a program developed by M.Rogers based on the algorithm of Brendel and Trifonov (1984) compares the test DNA sequence with a control array of known E.coli terminators and produces a numerical index of the closeness of fit between potential terminators in the test sequence and known terminators. This program identified 3 potential terminators within the region 3000-3500bp of the pBR322 map located at 3040, 3099, and 3157 with scores of 4.151, 3.222 and 3.693 respectively. These calculated locations agree closely with the inferred locations mentioned above. It is likely therefore, that the Z-Term positions are correct.

These predicted terminators are located very close to the region of DNA encoding RNAI and RNAII (3' end of RNAI and 5' end of RNAII) and it is possible that transcription readthrough past them disrupts the base-

pairing of RNAI to RNAII and causes increased synthesis of the pre-primer RNAII. This would result in elevated copy number and may interfere with Rom mediated catalysis of the RNAI/RNAII interaction. The absence of a bla gene in ColE1 upstream of this region means that strong transcriptional readthrough into the replication origin will not occur and consequently RNAI/RNAII interaction will proceed unhindered and plasmid copy number will be efficiently controlled.

In conclusion, the enhancement of RNAI/RNAII binding by Rom will depend upon the intrinsic "affinity" that particular RNA species have for one another (i.e. mutant or wild-type) in addition to the effect of outside influences on this region.

(ii) Why is pUC8 Copy Number So High.

The copy number of pUC8 should be similar to pAT153 (23 copies per genome equivalent) since both plasmids are rom⁻ pBR322 derivatives, but instead, pUC8 has a copy number of 87/genome equivalent. Sequence analysis revealed that EMS mutagenisis used to destroy restriction recognition sites during construction of pUC vectors had not introduced new lesions into their replication origins (Yanish-Perron 1985). Consequently, the replication origins of pBR322, pAT153 and pUC8 are identical.

It is difficult to reconcile this with the high copy number of pUC8. Tomizawa and Som (1984) demonstated that some mutations within the RNAI/RNAII coding region result in plasmids with very high copy numbers which can be reduced 3-7 fold by the action of Rom. Since pUC8 has a high copy number which is reduced 5-6 fold by Rom, it seems feasible that this vector does have alterations in the nucleotide sequence which were missed during sequence determination. However, if the published sequence is indeed correct, it can only be assumed that the transcriptional environments in pAT153 and pUC8 are sufficiently different to explain the copy number discrepancy and efficiency of Rom activity.

(iii) Aspects of Rom.

In the naturally occurring plasmid ColE1, the rom gene is transcribed towards oriV. This work has shown however, that its orientation with

respect to the replication origin in pUC8 is unimportant for both copy number control by rom and for stabilization of the plasmid. Moreover, this work has also demonstrated that rom reduces copy number and stabilizes pUC8 in trans. "Trans-stabilization" by Rom does not support the view that it acts in a "par-like" manner to stabilize plasmids, since regions of plasmids which confer stability in this way are cis-acting and there are no reports of par sequences affecting plasmid copy number e.g. pSC101par; R1stb (Meacock and Cohen 1979; Miki et al 1980). In addition, all plasmids reported to have a "par" sequence have low copy numbers. Plasmid pSC101 has a copy number of 5 per chromosome equivalent (Cabello et al 1976) R1 5-6 copies per cell (Nordstrom et al 1980) and F, 1 copy per genome equivalent. It is generally assumed that because of their low copy numbers, these plasmids cannot rely upon random segregation to ensure stable maintenance, but instead require an active partitioning mechanism. Since the rom gene cloned into pUC8 was derived from pBR322, a high copy number plasmid (derivative of pMB1), it is unlikely that these replicons would require an active partitioning mechanism to ensure stable maintainance.

The ability of a rom$^+$ plasmid to multimerise in a hyper-recombinogenic host JC8679, indicated that the mechanism of Rom action was dissimilar to that of the cer region of ColE1; parB of CloDF13 and lox of P1 (Summers and Sherratt 1984; Hakaart et al 1984; Hoess et al 1982). These DNA regions increase the stability of plasmids by resolving multimers to monomers, thereby maximising the number of segregating units available to be randomly partitioned. The cis-acting nature of ColE1 cer, in addition to the lack of involvement of cer in copy number control, suggests that the role of Rom in plasmid stability is totally unrelated to any "cer-like" activity.

(iv) How Does Rom Stabilize pUC8?

Several hypotheses, variance, clumping and sequestration have been proposed to explain the instability of the high copy number pUC8 plasmid. These arguements were extensively discussed in chapter 3 to explain the instability of plasmid multimers. Firstly, variance in plasmid copy number due to an inefficient replication control system, could partly explain pUC8 instability. If the control system of a plasmid reacts "sluggishly" to copy number deviations, then at cell

division the number of plasmid copies present in newborn cells may not be efficiently monitored. This could lead to over-replication in some cells and under-replication in others which may continue unchecked for several generations. If the control system does not respond to downward copy number deviations, subsequent cell divisions will generate plasmid free segregants. This plasmid has one of the two controlling elements possessed by naturally occurring ColE1-like plasmids and clearly, the RNAI/RNAII interaction in pUC8 cannot maintain the copy number at levels similar to pAT153, a closely related *rom*$^-$ cloning vector. This indicates that in pUC8 in the absence of Rom, this interaction fails to effectively control copy number. The ability of Rom to enhance RNAI/RNAII binding has been demonstrated by Tomizawa and Som (1984). Moreover, the level of RNAI required to bind to RNAII *in vitro* is lower in the presence of the Rom protein (Lacatena *et al* 1984) and *rom*$^+$ plasmids are maintained at lower copy numbers than their *rom*$^-$ counterparts. Taken together, these results suggest that there will be greater negative control by RNAI in *rom*$^+$ plasmids and the control system will be more responsive to copy number fluctuations.

Rom could act as a "fine tuner" of replication by increasing the sensitivity of RNAI to deviations from the mean copy number, so that they are promptly recognised and corrected. The molecular basis of Rom action is unclear, but it could achieve increased sensitivity by shortening the half-life of RNAI in addition to enhancing its binding rate to RNAII.

Low copy number variance could account for the stability of the *rom*$^+$ plasmid pCJ112 since its copy number would not fluctuate significantly from the mean value of 14 per genome equivalent (Table 6.1) which is adequate to ensure stable maintainance. In contrast, large copy number variance in a poulation of *rom*$^-$ pUC8 containing cells, would result in instability of this high copy number plasmid.

Unfortunately, neither of the methods discussed in results sections 6.7 (i) and (ii) produced any conclusive data on copy number variance. A third method which could be used to detect variance involves the use of a pUC8 compatible plasmid pTF464, which carries the ColE1 RNAI gene under the control of the lambda P_L promoter (B. Polisky pers. comm). At the non-permissive temperature, in a temperature sensitive CI857 host strain, RNAI is produced in large quantities from the induced lambda P_L promoter. Since there is a gene dosage dependent effect on copy number

with RNAI (Moser and Campbell 1983), pUC8 replication should cease and continued cell division will dilute out the plasmid copies. Using this system rather than a *polA12*$_{ts}$ host as a method to switch off plasmid replication may produce conclusive data on copy number variance in individual cells. The success of this experiment would depend upon how effectively RNAI alone can switch off pUC8 copy number. It is conceivable that in the absence of Rom, RNAI can "turn-down" plasmid replication but is unable to cause replication to cease completely.

A second possible explanation for the instability of pUC8 is the concept of plasmid "clumping". If plasmid copies within a cell aggregate together, either due to incorrect events at termination of replication, or due to some "molecular glue", the number of segregating units will be reduced. When this number falls below a critical level required for stable maintenance, random partition will result in the generation of plasmid free cells. All copy number estimation methods currently used, i.e. single cell resistance to antibiotics; hybridization assays and *in vivo* labelling techniques, measure the amount of plasmid DNA either directly or by inference from *bla* gene dosage (s.c.resistance to antibiotics) but none reveal anything about the location within the cell or state of catenation of the plasmids.

The involvement of DNA gyrase in decatenation of the *E.coli* chromosome has been demonstrated and its possible role in bacterial chromosome segregation has been discussed (Steck *et al* 1984). Similarly Wolfson *et al* (1982) have shown using either temperature sensitive *gyrB* mutants or gyrase B specific inhibitors that the maintenance of several plasmids is severely affected by antagonism of the B subunit of DNA gyrase. The authors speculate upon the role played by gyrase in plasmid maintenance and the requirement for supercoiled templates for initiation of DNA replication and for decatenation of plasmids are thought to be the main functions of gyrase subsequent to plasmid replication.

If replication of the *rom*$^-$ pUC8 molecule is relatively uncontrolled, it is possible that faulty termination may occur and catenanes may be formed in some cells. Presumably there is a limited intracellular pool of functioning gyrase and a plasmid such as pUC8 with a very high copy number will compete with the bacterial chromosome for the gyrase. In this situation, decatenation of plasmids may not occur and plasmid instability could result due to a reduction in the number of segregating units.

One observation would argue against catenanes being solely responsible for the instability of pUC8. A catenane composed of two plasmid units would run in an equivalent position to a monomer open-circle or plasmid dimer (M.Boocock pers. comm). Since single colony gel analysis of pUC8 monomer containing cells (DS903) show a large monomer band with a faint open circular band, it seems implausible that pUC8 instability can be solely attributed to catanane formation. Alternatively, plasmid aggregation, could occur via "connector" molecules e.g. RNA or protein. The harsh preparative treatment of cells prior to single colony gel analysis may disrupt any plasmid-plasmid interactions of this sort. Perhaps electron-microscopic analysis of DNA carefully isolated from pUC8 and pUC8*rom*$^+$ (pCJ112) containing cells would determine if aggregation of plasmid molecules occurs in high copy number *rom*$^-$ plasmids.

The final results section in this chapter dealt with the possibility that that competition between p^- and p^+ cells may account in part for the gross instability of pUC8 in DS903. Assuming that no competition occurs, the segregation frequency required to produce the final $p^-:p^+$ ratio seen in the mixed culture experiment (Fig.6.16A) would have been greater than that demonstrated in stability experiments. This suggests that competition must contribute to the outcome of the mixed culture experiment.

The lack of suitably marked host strains prevented the detection of p^- segregants which may have arisen during the course of the competition experiment. It would perhaps have been more profitable to construct via P1 transduction, a strain otherwise isogenic to DS903 apart from a phenotypically silent mutation in a sugar utilization pathway (other than glucose metabolism). Adams *et al* (1979) described the use of the *ara*Ah9 mutation as a suitable genetic marker for studying the segregation aspect of competition experiments. They demonstrated that the "fitness" of the *ara*$^-$ strain otherwise isogenic with the parental strain, was identical under the growth conditions used (glucose minimal medium). This suggests that by careful choice it may be possible to construct differentially marked bacterial hosts having the same growth characteristics as the parent strain for use in determining how much p^- segregants contribute to the outcome of a mixed culture experiment.

Most research on competition between plasmid free and plasmid containing cells has been carried out in chemostat culture using ColE1

derivatives (Jones et al 1980; Helling et al 1981; Wouters et al 1980; Noack et al 1981) and R factors. (Melling et al 1977; Godwin et al 1979). Generally, competitiveness between plasmid free and plasmid containing populations was greatest in environments where the nutrient restriction was severe (phosphate or glucose limitation). Very few competition studies have been carried out in batch culture possibly because most interest in the subject of differential growth rates between p^+ and p^- cells concerns large-scale fermentation processes. Dale and Smith (1979) however studied the effect of an R factor on host growth in batch culture. From their data they concluded that in batch culture, any competitive advantage of p^- cells over p^+ cells would manifest itself in the pre-stationery phase when nutrients become limiting. This may be reflected in the results from the DS903/DS903 pUC8 competition experiment since the difference in growth rate did not become apparent until late log phase. In contrast, no significant difference in growth rate in mixed culture between DS903 pCJ112 and DS903 was observed at any time during the growth curve. Presumably the metabolic load conferred on the host strain by the low copy number pCJ112 plasmid is negligable.

In retrospect and had time allowed, a more accurate estimation of pUC8 segregation frequency may have been produced if p^+/p^- assays had been carried out after shorter generation time intervals. This would circumvent the problem of competitiveness occurring in late log /early stationary phase due to nutrient limitation.

Irrespective of whether p^- segregants from pUC8 containing cells contributed significantly to the final outcome of the competition experiment, it is clear that p^- segregants arise during pUC8 stability experiments possibly due to copy number variance allowing competition effects to emerge.

CHAPTER 7

GENERAL DISCUSSION.

The aim of this thesis was to investigate the factors influencing the hereditary stability of multicopy E.coli plasmid vectors. Since their natural parental plasmids are stably maintained, the instability observed for plasmid vectors must be a consequence of deletions and rearrangements during their construction.

This work presented evidence that multimerization of pBR322 and pAT153 by generalized recombination, led to a progressive reduction in copy number, suggesting that an origin-counting mechanism existed. Concomitant with the reduction in copy number was a decrease in stability, indicating that random partitioning rather than an active mechanism was employed.

The complete DNA sequences of these plasmids were compared to that of ColE1, closely related to their parent plasmid, pMB1. Both pBR322 and pAT153 lack the cer region responsible for the breakdown of multimeric molecules. A similar determinant has been demonstrated in ColK and CloDF13; a conserved "core" sequence has been detected in ColE1, ColK, CloDF13 and pMB1, indicating that all of these multicopy plasmids encode a similar site-specific multimer resolution system (Summers and Sherratt 1984; 1985; Summers et al 1985 in press; Hakkaart et al 1984).

The contribution of competition between p^- and p^+ cells to the level of instability was highlighted by the experiments with pUC8 and pCJ112 in Chapter 6. Cloning the rom gene into pUC8 reduced its copy number 5-6 fold and yet dramatically increased stability, an apparently paradoxical observation since high copy number for randomly partitioned plasmids is generally sufficient to ensure stability. Subsequent analysis of stability and competition data for pUC8 and pCJ112, revealed that for pUC8, competition between p^- and p^+ cells accounted in part for the high level of instability. Therefore, especially for high copy number plasmids, it is unreasonable to assume that segregation alone is responsible for the levels of instability observed. It is pertinant to stress that the "segregation frequencies" measured in this thesis includes the contribution due to competition between plasmid-containing and plasmid-free cells.

The failure to obtain conclusive data on copy number variance is disappointing but unavoidable since with current technology, one cannot determine the plasmid copy number within individual cells at cell

division. In addition to reducing the competition between p^- and p^+ cells, the stabilizing effect of rom on pUC8 (pCJ112) could result from a reduction in copy number variance due to more efficient copy number control. This attractive idea is as yet untestable.

Since multimer instability is probably a direct consequence of a reduction in the number of segregating units, it was assumed that the instability could be counteracted by the use of par, an active partitioning mechanism from the low copy number pSC101 plasmid. par efficiently stabilized monomeric plasmids but failed entirely to stabilize multimeric forms of both pAT153 (pST2), pUC8 and pUC9. This demonstrates that par cannot be considered to be a "stability cassette" which is capable of functioning in any location on diverse plasmids. Further study on the efficacy of par in its natural location on multimeric pSC101 molecules would reveal whether the inability to stabilize multimers was peculiar to pMB1 derivatives.

In conclusion, the assumption that plasmids comprise functional segments interspersed with "junk DNA" which can be relocated at will during construction of cloning vectors, is naive. Prior to construction of optimal cloning vectors with respect to plasmid stability, the following points should be borne in mind. The replication origin should be "protected" from strong exogenous transcripts which could interfere with replication of the vector (Stueber and Bujard 1982; Chapter 6 concluding remarks). To minimise the competitive advantage of plasmid-free over plasmid-containing cells, the ideal vector should possess an intermediate copy number, sufficient to ensure its stable inheritance by random partitioning, yet low enough to prevent large competition effects between p^+ and any p^- cells which may arise. Also, the inclusion of rom in vector constructs may ensure minimal copy number variance, and reduce the probability that plasmid-free segregants would arise. The cer determinant should also be included in the construct to maximise the number of segregating units at cell division, particularly in recombination proficient hosts. In a multicopy plasmid construct containing all of the above features, par should be superfluous, but may be useful in specialised low copy number plasmids required for expression of factors which are lethal to the cell when produced in high concentration.

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