# PLASMID SEGREGATIONAL STABILITY IN *ESCHERICHIA COLI*.

by

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A thesis submitted for the degree of Doctor of Philosophy
in the University of Warwick
January 1984

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## RESULTS AND DISCUSSION - Part I

## Acknowledgements.

I am indebted to the Dept. of Biological Sciences, University of Warwick for providing the facilities necessary for the research described in this thesis and to Dr. S.B.Primrose for advice throughout the course of the work. In addition, I thank Dr.A.Robinson and Dr.D.C.Ellwood for advice during my time at Porton Down and the Science Research Council for providing the necessary funding.

I am especially grateful to Dr. Nick Mann for his advice during the preparation of this thesis and to Kay Pollitt for rendering the manuscript legible.

Finally I thank my wife and family for their continued support.

## Declaration.

The work described herein was done at the Department of Biological Sciences, University of Warwick and the Public Health Laboratory Service, Porton Down, whilst I was the recipient of a CASE award from the Science Research Council.

The experimental work described is entirely my own and has not been submitted for any other degree at any other university.

## SUMMARY

The technique of continuous culture has been used to study the segregational stability of plasmids in *Escherichia coli* in the absence of selective pressure.

Conditions were established that allowed the detection of plasmid-free cells no matter how low their initial frequency. Using these conditions the segregational stability of two related multicopy plasmids (pDSII09 and pBR322) was examined.

pDSII09 was found to be stably inherited throughout I20 generations of nutrient limited growth despite the observation that the plasmid copy number fell 4 to 5 fold during the culture period. By contrast, pBR322 was lost from chemostat culture after a lag of between 30 and 40 generations, a period during which (by analogy with pDSII09) its copy number had fallen about 2 fold.

The functional basis of the differential segregation exhibited by these plasmids was ascribed to the presence (on pDSII09) or absence (on pBR322) of a functional *par* (partition) signal that ensured the efficient segregation of plasmid molecules into daughter cells at division. Based on this hypothesis, experiments were done to examine the possibility for correction of the defective partitioning of pBR322 by complementation *in cis* and *in trans*.

In only two cases (both *in cis*) was complementation achieved. The first using a previously characterised *par* function from plasmid pSCIOI and the second using a fragment of plasmid pDSII09 that was considered (by argument) to be the region involved in its observed segregational stability.

Supportive evidence for the existence of partition elements amongst multicopy plasmids is cited, as is confirmatory work since published by other workers. A possible mechanism of *par* action is discussed.

Chemostat culture has also been used to examine the possibility of plasmid transfer by transformation within the chemostat. This study examined the effects of both growth rate and nutrient limitation on the transformability of *Escherichia coli* grown in continuous culture. The results obtained are discussed in relation to previously published transformation work using batch grown cells and a possible mechanism of plasmid transformation is suggested.

# Introduction

# I Bacterial Plasmids

## i) General Considerations

With the postulate by Lederberg (1952) that "extra-chromosomal hereditary determinants" existed in *E. coli* that were able to pass from a 'donor' cell to a recipient thus confering upon it new phenotypic traits, the notion of plasmids was born. At first, study of such new hereditary units was limited to the agent responsible for gene transfer between strains of *E. coli* (the fertility or F factor) and the existence of plasmids within *E. coli* was considered of academic interest only (Hayes, 1968; Lewin, 1977; Broda, 1979).

In the early 1960's however a parallel was drawn between the behaviour of the F factor in populations of *E. coli* and the spread of multiple antibiotic resistance markers throughout populations of the dysentry bacillus *Shigella* during outbreaks of the disease in Japan (Watnabe, 1963). Subsequent experimentation revealed that indeed the F factor and the *Shigella* antibiotic resistance (R) factors were different manifestations of a similar phenomenon (for documentation of the supportive work see Lewin, 1977; Broda, 1979); A replicating unit of double stranded circular DNA encoding, in addition to the ability to replicate and maintain itself, a variety of other phenotypic traits.

With the realization that plasmids encoded easily identifiable genetic markers, in particular antibiotic resistance, plasmid biology took a great leap forward. Moreover, improved methods for the isolation and visualization of plasmid DNA (e.g. Meyers *et al*., 1976) led to the

| PLASMID | SIZE(Mdal) | COPY NUMBER | PHENOTYPE CONFERRED ON HOST CELL |
|---|---|---|---|
| RI | 62 | I-2 | Tra,Ap,Cm,Km,Sm,Sp, Su. |
| R6 | 65 | I-3 | Tra,Cm,Km,Nm,Pm,Sm, Su,Tc. |
| RP4 | 36 | I-3 | Tra,Ap,Km,Tc. |
| pSCIOI | 6 | 5-6 | Tc. |
| CloDFI3 | 6 | IO-I2 | Cloacin production. |
| ColEI | 4·2 | IO-I5 | Colicin production. |

Table I. A selection of plasmids maintained in *E.coli.*

Key : Ap - Ampicillin resistance.

Cm - Chloramphenicol resistance.

Km - Kanamycin resistance.

Nm - Neomycin resistance.

Pm - Paramomycin resistance.

Sm - Streptomycin resistance.

Sp - Spectinomycin resistance.

Su - Sulfonamide resistance.

Tc - Tetracycline resistance.

Tra - Ability to mediate conjugation.

discovery of plasmid molecules in almost every bacterial species examined. In addition, analogous elements appear to exist in several eucaryotic species (Bea le and Knowles, 1978).

The variety of biological functions ascribed to plasmids is only matched by their heterogeneity in terms of size, copy number, mode of replication and host range and a small sample of this variety is presented in Table 1. A more complete listing of the plasmid/host combinations catalogued recently may be found in Bukhari et al. (1977).

ii) General Classification: Replication

The variety of plasmids present within any one bacterial genus, for example *Escherichia* had led to difficulties in their classification (Novick et al., 1976). Nevertheless, classification (of sorts!) has been achieved based on one property shared by all plasmids; replication.

Every plasmid that is able to exist within a population must encode the necessary information for its own replication, that is to say it must satisfy the requirements of a replicon (Jacob et al., 1963). Furthermore, plasmid replication must, to some degree, be linked to the replication of the host chromosome; the successful existence of a plasmid demands a balance between replicating continually and 'overriding' chromosome replication (as do most viruses) and replicating too slowly such that the plasmid is continually diluted out of a growing bacterial population.

Within these extreme boundaries, a plasmid can exist within a cell at anything between 1 copy per chromosome equivalent, to greater than 50 copies and the way in which plasmids control their copy number has been used for their classification (Novick et al., 1976).

'Stringent' plasmids are those whose replication is obligatarily linked to that of the host chromosome, when bacterial genome replication is stopped by the addition of a drug or starvation for an essential component, plasmid replication is also arrested. This similarity in behaviour reflects a mode of plasmid replication that shares some if not all of the components of chromosomal DNA replication.

By contrast, 'relaxed' plasmids are those whose replication is not so tightly linked to that of the bacterial chromosome; inhibition of chromosomal replication in this case does not necessarily prevent further rounds of plasmid replication.

Fortuitously, several other plasmid properties align themselves with the classification based on mode of replication. Firstly size; in general, large plasmids exhibit the stringent mode of replication whereas small plasmids exhibit the relaxed mode. Secondly, copy number which is to some extent linked to plasmid size (Dougan and Sherratt, 1977), tends also to be a reflection of mode of replication, stringent plasmids being of low copy number and relaxed plasmids of high copy number. Finally, the ability to mediate self-transfer (conjugation) is also a property almost exclusively found amongst large (and consequently stringent low copy number) plasmids mainly as a consequence of the number of gene products required for this process.

Inevitably there are exceptions. Plasmid R6K for example (Crosa *et al.*, 1976) is a large multi-antibiotic resistance plasmid that exhibits high copy number, a relaxed mode of replication and the ability to conjugate, whilst plasmid pSC101 (Cohen and Chang, 1977) is a small tetracycline resistance plasmid that, although non-conjugative, exists at relatively low copy number.

Interestingly, although small plasmids do not encode the necessary information to mediate their own conjugal transfer many are able to utilize the conjugal machinery of other large plasmids if present in the same cell. Thus the small colicin factor ColE1 can be transferred to $col^-$ *E. coli* if the F factor is present in the same cell. This process is termed mobilization and is a common property of many small naturally occurring *E. coli* plasmids (Warren *et al.*, 1978).

iii) Specific Classification: Incompatibility

The mode of plasmid replication provides the basis for a further level of classification within either the 'stringent' or 'relaxed' group. Plasmids can be grouped together on their ability to co-exist with one another in the same bacterial cell, a property referred to as 'compatibility'.

When two plasmids (A and B) are introduced together into the same cell, one of two outcomes can occur in subsequent generations of non-selective growth. 1) Both plasmids can exist stably together and be maintained within each cell of the population, or 2) the original dual plasmid containing cell can segregate A-only or B-only progeny that grow further to give a mixed final population.

When situation (1) is the result of such a test, the two plasmids under study are said to be compatible, whereas situation (2) is the result when incompatible plasmids are present in the same cell.

Incompatibility defined in this way is a measure of the relatedness of the regions of copy number control present on any two plasmids and their competition for membrane sites involved in partitioning. There seems little doubt that, in most cases, the repressor dilution theory of replication control put forward by Pritchard *et al.* (1969) is the favoured model for the control of plasmid copy number (e.g. Uhlin and Nordstrom, 1975; Cabello *et al.*, 1976; Warren and Sherratt, 1978; Shepard *et al.*, 1979) and all observations on plasmid incompatibility are consistent with this model.

When two plasmids whose replication repression molecules are sufficiently similar to cross-react are present in the same cell, replication of each is blocked until the total number of copies of the two together falls to the value normally observed when only one is present. This situation is clearly unstable and results in descendants that have lost one or other of the two plasmids. Multicopy plasmids exhibit the same phenomenon although the number of generations required before differential segregation of plasmids occurs may be higher (Warren and Sherratt, 1978; Hashimoto-Gotoh and Timmis, 1981).

The discovery of incompatibility amongst small multicopy plasmids, coupled with the current knowledge of their regulation of copy number

(Shepard *et al.*, 1979; Twigg and Sherratt, 1980; Muesing *et al.*, 1981) suggests that control of replication amongst such plasmids is every bit as detailed as that observed for large low copy number plasmids. It is worthy of note therefore, that the classification terms 'stringent' and 'relaxed', whilst providing a useful basis for grouping plasmids together, do not imply a 'tight' or 'loose' control over plasmid replication and copy number. They should only be used in accordance with the definitions previously described (Novick *et al.*, 1976).

Incompatibility has provided a useful bases of plasmid classification and most plasmids of the Gram-negative bacteria have been assigned to one or other of about 20 incompatibility (Inc) groups (Datta, 1976; Novick *et al.*, 1976). Whilst the criteria for such groupings is microbiological, consideration of its underlying basis implies that it is a largely natural classification based on evolutionary relationships between plasmids, in particular between their replication regions. Indeed, direct measurement of the DNA sequence homology between plasmids has confirmed that, in most cases, plasmids within Inc groups are more related than those from different groups (Guerry and Falkow, 1971; Grindley *et al.*, 1973).

iv) Plasmid maintenance and segregation

For a plasmid to be stably maintained within a bacterial population, two criteria must be fulfilled. Firstly, as described in a previous section, there must be sufficient replication of the plasmid to ensure at least one plasmid copy for each cell in the population. Secondly, there must be some method of ensuring the partition of newly replicated plasmid molecules into the progeny of dividing cells.

The need for such a specific assortment mechanism becomes apparent if the consequences of a purely random distribution pattern are considered. In the absence of any partitioning mechanism, the probability of either daughter cell failing to inherit a plasmid (P(o)) is a function of the plasmid copy number (C). The exact relationship is given by the binomial relationship:

$$P(o) = 2\ (\tfrac{1}{2})^{C}$$

In the case of a low copy number plasmid such as F, normally present at 1-2 copies per genome equivalent, the probability of generating a plasmidless cell can be as high as 0.5 (copy number of 2). Yet, despite this 1 in 2 chance of segregation very few F(-) cells are found in populations of F(+) bacteria (Anderson and Lustbader, 1975). On the basis of this observation, Jacob et al. (1963) suggested that a specific mechanism of F segregation must exist. They further suggested that attachment of the F factor to the bacterial membrane followed by migration of that coupling site into daughter cells at division could accomplish the inferred partitioning. A similar proposal was made for segregation of the bacterial chromosome itself, and was apparently substantiated by the findings of Worcel and Burgi (1972) that the folded chromosome of E. coli could indeed be isolated bound to the bacterial membrane. By contrast, Jones and Donachie (1974) suggested that although the chromosome was membrane bound during DNA replication, it was released from the membrane at the termination of DNA synthesis and prior to cell division. There is therefore no necessary linkage of membrane binding to segregation.

An alternate model for the efficient segregation of low copy number plasmids, in particular F, was suggested by Hohn and Korn (1969) based on their own work and that reported earlier by Cuzin & JACOB, (1967). They observed that when the F factor was 'cured' from bacterial cells by the action of acridine orange it preferentially segregated with the chromosome it was associated with in the ancestral cell, an observation that suggested an association between the chromosome and the F factor. The finding by Kline and Miller (1975) and Kline et al., (1976) that, under suitable conditions, 90% of the cellular F DNA could be isolated bound to the folded chromosome adds weight to this model.

Undoubtedly there is an association that ensures a high degree of efficiency in the segregation of F, and presumably other low copy number plasmids, into daughter cells just prior to their separation, how accurate this mechanism is, and how it may function will be discussed in later chapters.

Despite the wealth of suggestion (above) concerning the mechanisms of partition amongst large plasmids, their size and complex genome organisation has complicated exacting studies on the nature of the partition function. In smaller plasmids however, the relatively simple genome organisation allows for an easier analysis of partition and the possibility of delineating the minimum region required for efficient segregation. This has indeed proved possible for the small tetracycline resistant plasmid pSC101; Meacock and Cohen (1980) reported the definition of a small (270 base pair) segment of DNA situated close to the origin of replication that accomplished the active distribution of plasmid DNA molecules to daughter cells. This region of DNA was not directly involved in plasmid replication functions for, if tetracycline was present in the growth media, par$^-$ derivatives of pSC101 were stably maintained indicating that replication *per se* could continue. Moreover, the *par* locus could complement *in cis* (but not *in trans*) the defective segregation of other non-related replicons.

How could this small segment of DNA exert its action?

As noted by Meacock and Cohen, partitioning requires that the *par* function be able to orientate plasmid molecules with respect to the plane of cellular division. The *par* locus might act therefore by interaction with a cellular structure (the cytoplasmic membrane or the folded chromosome?) that effects the actual distribution of plasmids into daughter cells. In this regard, two recent findings merit discussion. Churchward *et al*. (1983) have described the entire nucleotide sequence of the replication region of pSC101 including the region encompassing the *par* locus of Meacock and Cohen. No open reading frames of significant length occur on either of the two DNA strands in this region, but a small region (11 base pairs) of DNA is repeated in such a manner as to allow the formation of a hairpin structure within the DNA duplex. In the absence of any other significant features, Churchward *et al*. suggest this small region of secondary structure as the region active in the partition system. Confirmation of this however must await further localization of the *par* locus.

The mechanism of *par* action has been recently discussed by Gustafsson *et* *al*. (1983) who have found that all $par^+$ plasmids including those containing the *par* locus of Meacock and Cohen, are found bound to the outer membrane of *E. coli* after fractionation of *E. coli* cell wall layers. This association is not observed when the cell wall layers of bacteria carrying $par^-$ plasmids are analysed. These results led Gustafsson *et al*. to suggest that, at least in the case of pSC101, binding of plasmid molecules to the membrane is the structural basis of the observed efficient partitioning.

The case for accurate partitioning systems amongst large low copy number plasmids seems strong, and in the case of a small intermediate copy number plasmid (pSC101) a region of DNA actively involved in partition has been precisely mapped and studied. The case for a specific segregation mechanism amongst high copy number plasmids however is much less clear. With a copy number of about 20 per genome equivalent, the calculated probability of a bacterial cell not inheriting the plasmid ColE1 is about 1 in $10^6$. With such low values it is questionable whether or not a system of segregation is warrented as the random distribution of plasmids within the cytoplasm of the dividing cell will ensure that most cells in a population inherit a plasmid. However, note that the calculation of probabilities involves an exponential function and, should the copy number of (for instance) ColE1 fall by only 2 fold, the probability of a plasmid-free cell arising within a population increases about $10^3$ fold!

The relationship between plasmid copy number, plasmid stability and the need for a specific partitioning system is dealt with extensively in the later chapters of this thesis.

## II Continuous Culture

### i) Discovery and Development

The requirements for growth of (for instance) a prototrophic strain of *E. coli* are extremely simple; a source of the essential elements of life (usually as mineral salts), an oxidisable substrate to provide energy for biological processes and a reducable substrate for the elimination of electrons that have already provided such energy. These prerequisites provide the minimal requirements for growth and it is clear that, in a situation where all requirements are present bar one, there can be no growth until the one limiting substrate is added.

It follows that, when a very small amount of a limiting substrate is added to an otherwise complete system, the amount of growth will be correspondingly small. By contrast, if the limiting substrate is present in far greater amounts, growth can proceed at its maximum possible rate until one or other of the essential components is again exhausted. In other words, the amount of growth possible is directly dependent on the amount of any limiting substrate present.

This reasoning forms the basis of two simultaneous, though independent reports published in 1950 describing techniques whereby a bacterial culture could be maintained in a state of constant and controlled growth.

The 'chemostat' (Novick and Szilard, 1950) and a similar device the 'bactogene' (Monod, 1950) were apparatus that, by limiting the concentration of one substrate when all others were present in excess,

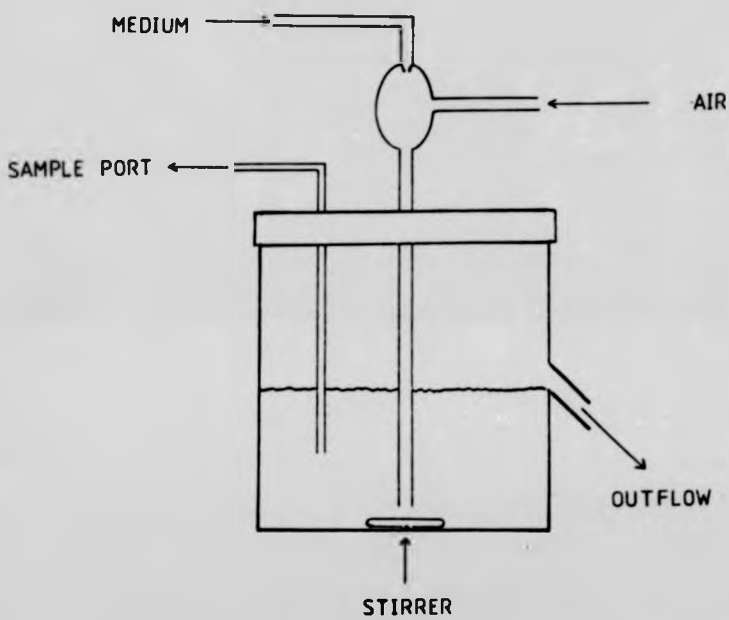



Figure I. A simple diagram of the essential components of the chemostat.

were able to control the growth rate of bacterial cultures. By the simple introduction of a device to ensure a controlled constant addition of media and an overflow for the removal of exhausted media and additional bacteria, a machine was developed that could allow the growth of any bacterial strain for an unlimited period of time.

In practice, todays chemostats are complicated by the addition of various ancillary devices that ensure complete control of the culture vessel itself. The principle behind its operation however remains the same; a controlled supply of sterile media in which one essential substrate is present at low enough concentrations to limit growth, is continually added to a culture vessel in which the strain under examination is grown. Aeration (if required) is supplied by a small pump and the culture is agitated vigorously to ensure good mixing. Ancillary equipment such as pH controllers, anti-foaming devices and temperature controllers maintain a constant bacterial environment, and a constant level device (usually no more than a side arm in the culture vessel) maintains a constant culture volume and provides an exit for exhausted media and bacteria. A typical chemostat is shown in diagramatic form in Figure 1.

ii) Theory of Growth and Competition

The mathematical basis of bacterial growth in chemostats was originally described by Monod (1950) and Novick and Szilard (1950) and has since been exhaustively reviewed and extended (e.g. Herbert et al., 1956; Tempest, 1969; Kubitshek, 1970; Dykhuizen and Hartl, 1983).

Only two basic relationships need concern us for the work described in this thesis. The first is arrived at by reason: consider a chemostat of the type in Figure 1 operating with a vessel volume of V ml and an input flow of media of F ml/hr. The organisms present within the culture will be diluted out of the vessel by the continual input of fresh media. Furthermore, the rate at which that dilution occurs is equal to $\frac{F}{V}$ and is given the symbol D (for dilution rate) and the units $hr^{-1}$.

Consider now, the growth of an organism within the culture vessel. So long as the dilution rate is set to a value that allows the organism to divide before being washed out of the vessel, then the population within the vessel will grow to a final cell density determined by the level of the limiting substrate in the incoming media. Growth, that is the rate of increase of organism concentration with time (given the symbol $\mu$) cannot proceed beyond the limit set by the concentration of limiting substrate but neither can it cease, for whilst the chemostat is in operation there is a continual inflow of fresh media.

Clearly, this situation must resolve itself in a perpetual state of limited growth, a steady state where the amount of growth ($\mu$) exactly balances the rate at which the vessel contents are replaced (D). In other words, under steady state conditions $\mu = D$.

This is the first key property of the chemostat, that, by altering the flow rate of incoming media (and thus the dilution rate) a population of bacteria can be kept indefinitely at a variety of growth rates from 0 to $\mu$ max. Under each of the possible growth rates the population density (being determined by the level of limiting substrate) remains the same, as do all external environmental factors. This unique system thus allows a direct comparison between growth rate and any other examinable trait. The ability of a cell to become competent for the uptake of DNA for example is a system that receives a good deal of attention in later chapters.

It has already been stated that the limitation of the rate of cell synthesis is achieved through the limitation of the concentration of a chosen nutrient. However, no exact relationship between these two values has been shown. A relationship does exist and, since nutrient uptake involves enzyme catalysed reactions it is not surprising that such a relationship resembles the Michaelis-Menton equation for enzyme rates.

The Monod (1942) equation states that:

$$\mu = \mu\,max \quad \frac{S}{Ks + s}$$

where $\mu$ = specific growth rate expressed in a nutrient limited chemostat.

$\mu$ max = growth rate that would be achieved if the limiting substrate were present in excess.

S = concentration of limiting substrate and,

Ks = saturation constant that is equal to the concentration of limiting substrate that allows growth to proceed at one half its maximal rate.

The relationship was found by Monod to hold true over a wide variety of real cultures and has been further substantiated by Herbert _et al_. (1956).

It has already been reasoned that when a chemostat is run with a dilution rate set below a critical value (equivalent to $\mu$ max), then a steady state is established where $\mu = D$. The Monod equation reveals that, under such conditions the growth rate is actually controlled by the concentration of the limiting substrate in the extracellular growth medium, a value that in turn is determined by the dilution rate. A full mathematical statement of these same conclusions has been published by Herbert et al., 1956 and Kubitshek, 1970.

Consider finally a situation where two strains of the same species (or two different species) for example with and without a bacterial plasmid are cultured together in a chemostat, two situations can be envisaged. First, both strains could replicate at equal rates such that their relative numbers stay the same throughout the chemostat run. Second and more likely considering the strains are different, competition could occur for the one limiting substrate resulting in a predominance of one or other of the two original strains. What would determine the outcome of such competition?

The Monod equation already described, would apply to each of the competing strains. As the level of S, the limiting substrate concentration is fixed in the chemostat vessel it will be the same for each strain. The strain that can attain the higher value of $\mu$ necessary to predominate in the competition is the strain that has either a higher $\mu$ max, a lower Ks or the best combination of both. These conclusions have been reached independently after rigorous mathematical treatment of the competition parameters by Powell (1958), Taylor and Williams (1975), Fredrickson (1977), and Slater (1979), and they hold true at any one growth rate provided that 3 criteria are upheld:

1) That no interactions occur between the two competing populations which are likely to modify or stabilize the kinetics of free competition.
2) That the growth environment remains constant throughout the course of competition, and
3) That both organisms respond to the growth conditions in an identical fashion.

Condition (2) above is one of the 'key' benefits of continuous culture and makes chemostats ideally suited to competition experiments (e.g. Bungay and Bungay, 1968; Veldkemp and Jannasch, 1972; Slater, 1979), moreover, the ability to maintain a mixed population for a large number of generations allows for very weak competition to eventually result in measurable population changes. Some examples of the use of continuous culture for competition experiments, especially those relating to plasmid stability within populations are described in more detail below.

## III Competition in Chemostats

### i) Types of selection

In any chemostat-maintained growing population, the frequency of mutation in any one gene will be the normal 'spontaneous' rate for the strain under study, usually $10^6$-$10^7$ per generation, and as long as that mutation does not directly affect either $\mu$ max or Ks it will be, in evolutionary terms, neutral. The number of such neutral mutations rises during a period of culture for, not only does each mutant divide to maintain its relative proportion within the population but inevitably the same mutation re-occurs elsewhere within the culture. The increase in apparently neutral mutations with time has been studied by several groups interested in the influence of such parameters as growth rate and nutrient limitation on the rate of mutation (e.g. Novick and Szilard, 1950; Kubitschek, 1974; Cox and Gibson, 1974) and has revealed that, as predicted, non-selected mutations rise in the chemostat linearly for periods of 50-100 generations. However, in all periods of culture so far examined, the rise in non-selected mutations does not continue indefinitely. After a period of (usually) 100-200 hr growth a mutation occurs within the culture vessel that directly affects the fitness of the growing strain. Such a mutant is selected for in subsequent generations and rises to predominate the culture at the expense of all other neutral mutations. The result is a rapid decline in the numbers of the neutral mutation under study to a basal level from where they once again begin to rise. This erratic and unpredicatable changeover in culture vessel populations has been termed 'periodic-selection' by Attwood _et al_. (1951), and likely represents an important mechanism of microbial evolution.

What kinds of mutation give rise to periodic selection?

As outlined in a preceeding chapter, any change increasing the $\mu$ max of the strain under study or lowering the Ks for the limiting substrate has the effect of rendering that strain 'fitter' in terms of chemostat survival, and so at a competetive advantage (Hartl and Dykhuizen, 1979). Moreover, as selection will be the end result of any favourable change in these parameters no matter how small, changes at many intermediate stages of metabolism could affect strain fitness. In the majority of cases where periodic selection has been observed, the genetic basis of change has not been determined but there are a few notable exceptions. Chao and Cox (1983) followed up earlier findings by Cox and Gibson (1974) that mutator strains (those strains with an enhanced rate of spontaneous mutation) of E. coli are selected for in the chemostat. Such strains are fitter because they have a greater chance of undergoing a favourable mutation (Nestman and Hill, 1973; Chao and Cox, 1983). Interestingly, one of the mutation loci that was selected for, mut Tl maps in an early region of the E. coli genetic map amongst a set of genes controlling cell division. Clearly the possibility exists that mut Tl itself or secondary effects caused by it influence the efficiency of the cell division process.

Other periodic selection events may occur in genes directly involved in the metabolism of the limiting substrate. Lactose-limited chemostats for example regularly give rise to mutants constitutive for the production of β-galactosidase (Novick and Horiuchi, 1961), and in phosphate-limited chemostats mutations in the yeast Saccharomyces cerevisiae arise at the locus of the acid phosphatase whose activity effectively limits growth (Hansche et al., 1978).

As a result of its unpredictability, periodic selection has proved a difficult phenomenon to study and as already noted, the genotypes of most selected strains are not known (Dykhuizen and Hartl, 1983).

Selection within the chemostat however, can be studied in more detail when two strains of known genotype are deliberately mixed within the chemostat. It has been proposed (Zamenhof and Eichorn, 1967) that in such cases auxotrophic mutants of any one strain will be at a selective advantage over their otherwise isogenic prototrophic counterparts as a result of the energetic economies achieved through fewer biosynthetic steps. This has been largely borne out in practice; His$^-$ and trp$^-$ auxotrophs of *B. subtilis* for example are selected for at the expense of their prototrophic counterparts in chemostats where the required amino acid is present in excess (Zamenhof and Eichorn, 1967; Dykhuizen, 1978), and tyr$^-$ auxotrophs of *E. coli* exhibit a similar selective advantage (Mason and Slater, 1979).

A similar situation occurs when mutants that overproduce an unnecessary metabolite are co-cultured with the wild type strain. Here, the mutant is at a disadvantage and is selected against during culture (Baichi and Johnson, 1968).

A further example of the selective advantage gained by energy conservation is the observation by James (1978) that mutants of the yeast *Saccharomyces cerevisiae* that have lost >95% of their mitochondrial DNA content are favoured at the expense of the wild type in anaerobic chemostats. This last example is especially relevant for it demonstrates the strain on cellular resources imposed upon a host cell by the presence of a non-essential extra-chromosomal DNA molecule; indeed there are a number of similar findings relating to plasmids in *E. coli*.

ii) Plasmid stability in populations

In environmental conditions that do not select for the functions encoded by a bacterial plasmid, the energy conservation hypothesis presented above predicts that loss of the plasmid will lead to a plasmid free (R-) cell 'fitter' than its R+ counterpart. This point has been made by several other authors (Chabbert *et al.*, 1969; Anderson, 1974; Lacey, 1975) and indeed, in many cases the presence of a plasmid has been shown directly to lower the growth rate of the host cell (Chopra and Lacey, 1975; Hershfield *et al.*, 1976; Inselberg, 1978; Zund and Lebek, 1980).

In some cases, the presence of a plasmid within a host cell has been reported not to change the growth rate by any measurable degree (Nordstrom et al., 1977; Cullum *et al.*, 1978) but these observations have been countered by Dale and Smith (1979) and Adams *et al.* (1979), who have pointed out that growth rate determinations in pure batch culture are seldom reliable indications of the behaviour of the strains concerned during competition in open (continuous) culture conditions. This last point suggests the utility of chemostats for the study of plasmid segregational stability. Chemostats offer a number of advantages over batch culture studies. First, they allow a limitless number of generations of growth under identical culture conditions. Second, chemostat theory predicts that, no matter how small the energy economy through loss of a plasmid the R- progeny will rise to dominate the culture; problems of detection of low numbers are therefore avoided. Third, chemostats offer the attractive possibility of allowing a study of nutritional parameters (growth rate, nutrient limitation) on plasmid stability.

These advantages have not gone unnoticed by other workers: Melling *et al*. (1977) used continuous culture conditions for the study of the stability of plasmid RP1 in the *E. coli* host W3110. They observed no loss of plasmid-borne markers throughout sixty generations of growth and concluded that RP1 was segregated stably under all conditions tested. In order to test the culture conditions for R- selection a small ( 1% v/v) inoculum of *E. coli* W3110 was added to a steady state culture of W3110 (RP1) under a variety of growth conditions. Competition and the eventual selection of the R- strain occured in both phosphate- and glucose-limited culture but was more pronounced under the former. No competition was observed under magnesium-limited conditions.

Similar results were obtained by Wouters *et al*. (1978, 1980) whilst studying the stability of plasmid R6 in *E. coli* PC0917. No loss of R6 was observed during several long periods of continuous culture in the absence of selective pressure implying again segregational stability. The ability of each culture to select R- populations was tested by the introduction of PC0917 and in concorde with Melling *et al*., selection was observed under phosphate-limited conditions and, additionaly under conditions of glucose limitation anaerobically; a result that led Wouters *et al*. to propose a state of effective energy limitation as the cause of selection.

The reports by Melling *et al*. (1977) and Wouters *et al*. (1978) indicated maintenance of the entire plasmid during extended periods of growth; no plasmid-borne marker was ever lost. This observation suggests that not only are plasmid RP1 and R6 segregationally stable but also that fragmentation of each plasmid is a rare event. This was not however the case for plasmid Tp120 whose segregational stability in *E. coli* has been studied by Godwin and Slater (1979). Under conditions of both phosphate- and glucose-limited growth plasmid Tp120 was observed to loose one or two of its four antibiotic markers. Although plasmid-free strains were not detected, each varient of Tp120 rose to dominate the culture in a similar manner to the R- strains of Melling and Wouters. Dissociation of markers is evidently a common event in *E. coli* strains carrying Tp120 and, after unequal partitioning the smaller of the dissociated forms is selected for in subsequent generations of growth.

Plasmids RP1, R6 and Tp120 are all examples of large complex plasmids that encode several identifiable genetic markers. In addition, all are able to undergo conjugal transfer and RP1 and Tp120 exist at relatively low copy number and are thus considered stringent plasmids (see section I(ii) of Introduction). The selection observed against such plasmids in competition situations in the chemostat also occurs however when the plasmid concerned is a member of the 'relaxed' group of plasmids typified by ColE1; Adams *et al*. (1979) and Imanaka *et al*. (1980) have both reported the loss of plasmid RSF2124 (a close relative of ColE1) within both continuous and batch-grown cultures. The R- strains so generated each had higher growth rates than the original plasmid bearing strains and rose to a high proportion of the final culture. Other small plasmids have also been lost upon repeated subculture; Falkow *et al*. (1977) showed that a small uncharacterized plasmid of the *Enterobacteriacae* was lost at different rates from different Gram negative hosts. The result is of interest for it demonstrates the contribution of host genetic background to the stability of plasmids within populations.

The stability of plasmids within populations is primarily a problem of academic interest; how does a plasmid ensure its survival within a population even when the genetic information it encodes is redundant? There is however a related applied concern; it is the stability of genetically engineered organisms that carry new genetic information on plasmids (many related to the plasmid ColE1). Information is lacking about both the stability of such recombinants in large fermenters designed to maximize the expression of a beneficial product and the fate of recombinant plasmids after accidental leakage into the natural environment. The problem has been neatly summarized by Neijssel (1980) and is exemplified by two simple examples.

Itakura *et al*. (1977) reported the successful construction of a plasmid that encoded the eucaryotic gene for the hormone somatostatin fused in phase to the gene for the bacterial protein β-galactosidase. However, when such a construct was grown even in small scale batch culture, 30% of the final population contained deletions in the *lac* operon and, by reference to the results of Godwin and Slater (1979) such deletion derivitives would have risen to predominate the culture if it had been grown indefinately. A similar observation was reported by Dwivedi *et al*. (1982), who studied the stability of a derivative of plasmid pSC101 that encoded the *E. coli* trp operon and was capable of producing large amounts of tryptophan in small scale cultures. When the process was scaled up however in an industrial fermenter a variety of deletion derivitives of the trp - pSC101 plasmid arose and quickly took over the culture to render it an uneconomic means of producing tryptophan.

These reports demonstrate the problems that can arise during large scale culture of plasmid bearing strains and indicate clearly that an understanding of the factors promoting plasmid stability would be of some benefit.

In summary, for reason of academic and applied interest, the problem of plasmid stability within populations seems worthy of pursuit. Moreover, the technique of continuous culture provides a powerful tool for such study for, not only can populations be maintained indefinitely in precisely controlled environmental conditions, but the influence of those conditions on the outcome of experiments can also be determined. Continuous culture also mimics to some extent a variety of natural microbial niches, for example the human gut (see Mason and Richardson, 1981) and as such, results obtained with chemostat populations may be directly applicable to environmental situations.

It is in the light of these considerations that research presented in this thesis was conceived and done.

This section deals with the materials and methods used during the course of the work presented in this thesis.

For ease of reference, techniques and recipies have been classed into one of three subsections.

I General methods of strain growth and storage

II Operation of the chemostat

III Techniques for the isolation, purification and subsequent manipulation of DNA.

## I General Methods

### a) Batch growth of cultures

All growth of bacterial strains not requiring defined media was done in L-broth (Miller, 1972). Liquid media was routinely sterilized by autoclaving at 15 psi (121°C) for 20 minutes.

Solid media was prepared in a similar fashion, but for the addition prior to autoclaving of agar to a final concentration of 1.5% (w/v).

Occasionally, 'soft' agar was required for the formation of bacterial lawns and for this purpose the concentration of agar was reduced to 0.6% (w/v).

For the selection of antibiotic resistant bacteria filter-sterilized antibiotics were added to solid or liquid media to the following final concentrations:

| | | |
|---|---|---|
| Ampicillin | - | 100 μg/ml |
| Tetracycline | - | 10 μg/ml |
| Kanamycin | - | 30 μg/ml |
| Chloramphenicol | - | 25 μg/ml |

Growth of strains in defined medium used the minimal salts base A.M.S. (Whittenbury *et al.*, 1970) and included any necessary amino acids and vitamins at final concentrations of 50 μg/ml and 5 μg/ml respectively. All cultures were grown at 37°C unless otherwise stated.

b) Strains

All bacteria used in this work were of the genus Escherichia. Relevant genotypes of each strain are described in experimental chapters. Strains were stored as either agar 'stabs' at 4°C or as suspensions at -20°C in a solution of glycerol (20% v/v) in L-broth.

c) Plasmids

Several plasmids are used in the course of this work and their origin and description is included in experimental chapters.

Plasmid-bearing strains were created by transformation and stored as described above. In many cases stocks of plasmid DNA were also stored as solutions in 10 mM Tris pH 8.0, 1 mM EDTA and frozen at -20°C.

d) Marker identification

For the routine analysis of large numbers of bacterial colonies for the presence of multi-antibiotic resistance plasmids replica-plating (Lederberg and Lederberg, 1952) was used. Occasionally, when replica-plating was inappropriate colonies were 'patched' onto the relevent antibiotic plates using sterile toothpicks.

e) Cell size and number

Cell numbers within growing populations were determined by colony counts after suitable dilution of the sample in isotonic saline (0.9% w/v NaCl). In some cases both cell number and size were monitored by the use of the Coulter Counter. Samples were suitably diluted in isotonic solution and counted under conditions recommended by the manufacturer.

f) Sucrose gradients

Cells were separated into different size classes by sedimentation through a neutral sucrose gradient varying from 5% to 20% sucrose in 0.1% phosphate buffer, pH 6.8. Sedimentation was for 15 minutes at 4000 rpm in a MSE Mistral 6L centrifuge. After sedimentation the gradient was fractionated from the top.

## II The operation of the chemostat

a) Apparatus

The chemostat used for most of the studies reported in this thesis was based on the commercially available 'Bioflo' chemostat (New Brunswick Scientific), the working culture volume of which was about 360 ml. The culture vessel with connecting tubing and pH probe were autoclaved as a unit and contamination was rarely a problem. Media was supplied via a separate peristaltic pump from a reservoir of approximately 10 L. Temperature was controlled via a thermostatic immersion heater that was designed for use with this apparatus.

b) Media

For much of the work described in this thesis two media were used, that intended to limit cell growth rate by the availability of carbon (as glucose), and that intended to limit cell growth by limiting the supply of phosphate (as phosphate buffer) in the incoming media. For both media the basal salts components were those described for A.M.S.

For glucose limited culture the glucose concentration was 0.2 gm/L, in all other (glucose sufficient) cultures the concentration was increased to 5 gm/L.

Phosphate was supplied as 0.1 M phosphate buffer (pH 6.8) to a final concentration of 5% (v/v) but was reduced to 0.01% (v/v) for phosphate-limited cultures.

For other limitations, the basal media A.M.S. was adapted in the following ways:

For nitrogen limited cultures the $NH_4$ Cl concentration was reduced to 5 mg/L and 1 gm of NaCl per litre was added.

For $Mg^{++}$-limited cultures the concentration of Mg $SO_4.7H_2O$ was replaced by 1 gm of $(NH_4)_2$ $SO_4$ and 1 mg Mg $Cl_2$ per litre.

For sulphate-limited cultures the amount of Mg $SO_4.7H_2O$ was reduced to 1 mg/L and 1 gm of Mg $Cl_2$ per litre was added.

Media was autoclaved in 10 L quantities and sterile phosphate and glucose added to the necessary concentrations when cool. Auxotrophic requirements were present at the same concentration as that declared earlier.

c) Growth Conditions

During operation of the chemostat the pH within the culture vessel was automatically maintained at pH 6.8 by the addition of 2 M NaOH. The incubation temperature was 37°C unless otherwise stated and the culture was kept aerated with a stream of sterile air dispersed by the rotation of a bladed magnetic flea seated in the culture vessel.

d) Sampling

Samples of bacteria were withdrawn via a sample port, connected to the main culture vessel, into a precooled glass vessel and kept on ice until used. Alternatively, samples were mixed with cold glycerol to a final concentration of 20% (v/v) and stored frozen at -20°C until required.

e) Growth Rate

Control of the growth rate within the growing culture was achieved by the control of the flow of incoming media via a variable peristaltic pump. The flow rate was measured by timing the usage of media from a graduated pipette situated before the pump. This value (in $ml.hr^{-1}$) was used to calculate the DILUTION RATE of the culture from the formula:

$$D = \frac{F}{V}$$

where D = dilution rate ($hr^{-1}$)

F = flow rate ($ml.hr^{-1}$)

V = working volume of the culture vessel

Under the conditions of limited growth generated in a chemostat where all nutrients necessary for growth are present in excess bar one (the limiting substrate) then when the culture achieves 'steady state' the value of D is equal to $\mu$ (specific growth rate).

f) <u>Assay for limiting state</u>

Phosphate in the media of cultures growing under phosphate limitation was assayed by the method of Deuel *et al*. (1970).

For glucose limited culture the availability of free glucose in the culture fluid was monitored by use of the commercially available 'Clinistix'.

For other limitations, limitation was checked only qualitatively. A sample of limited culture was removed from the chemostat and divided into two aliquots. One sample then received the limiting substrate in excess, the other an equivalent volume of saline. Both cultures were incubated for several hours at 37°C with shaking. At the end of this period the $OD_{540}$ was taken for each sample, and an increase in the relative cell density of the sample that had received the limiting subtrate in excess was taken as indicative of an effective limited culture.

III Manipulation of DNA

a) Preparation of plasmid DNA

Plasmid DNA was routinely prepared in two ways. 'Mini' plasmid preparations were analytical plasmid extractions from only 10 ml of L.B. The method of Birnboim and Doly (1979) was used throughout this work for extractions of this scale.

For large preparative DNA preparations, cultures containing plasmids were grown in about 300 ml of L.B. and amplified by the addition of chloramphenicol (150 μg/ml) if applicable. Chloramophenicol blocks protein synthesis and thus stops the further replication of the *E. coli* genome. Plasmid replication however can continue for it depends solely on proteins already present in the cell. After such treatment plasmid numbers may increase to several thousand per cell (Clewell, 1972). Lysis of such large scale cultures was by the 'cleared lysate' method of Clewell and Helinski (1972).

Susbsequent purification of plasmid DNA away from contaminating RNA and protein was by chromatography on hydroxyapatite as described (Colman *et al.*, 1978).

b) Plasmid Analysis

Plasmid DNA isolated as described above was analysed in agarose gels made and run in a buffer containing 0.09 M Tris-borate, pH 8.3, 2.5 mM EDTA and 0.5 μg/ml ethidium bromide. DNA bands were visualized by illumination of the gel with UV light and photographed with a Polaroid

Land camera through an orange filter.

Occasionally, it was necessary to concentrate or desalt DNA preparations by precipitation. This was achieved by the addition of sodium acetate to a final concentration of 0.3 M followed by 2.5 volumes of cold ethanol. The mixture was chilled to $-70^{o}C$ in a dry-ice ethanol bath for 15 minutes and the DNA recovered by centrifugation at 12000 rpm for a further 15 minutes. The final pellet was rinsed in ethanol, dried and resuspended in 10 mM Tris, pH 8.0, 1 mM EDTA.

Further analysis of plasmid DNA was achieved through the use of restriction endonucleases under the conditions recommended by the vendor. Generally, about 1 µg ($A_{260}$ = 0.02) of DNA was digested in each reaction and the products were analysed by agarose gel electrophoresis as described.

In order to create new plasmid molecules, DNA fragments created by restriction enzyme digestion were ligated together using the enzyme T4 DNA ligase. Ligation was carried out in a buffer recommended by the vendor for 16 hr at 15 $^{o}C$. Ligation products were identified after transformation of the ligation mix into E. coli.

c) Transformation of E. coli

The transformation procedure used in this work was based on that reported by Humphreys et al. (1979) to induce maximum competence in E. coli. After the induction of competence by $CaCl_2$, 100 µl of the cell suspension was incubated with the DNA sample (10 µl or less) at $0^{o}C$ for

20 minutes. The mixture was transferred to a water bath at 37°C for 10 minutes and then diluted 2 fold by the addition of prewarmed L-broth. After a further 90 minutes at 37°C transformants were selected by spreading aliquots of the mixture onto antibiotic agar plates followed by overnight incubation.

The transformation frequency was expressed as the number of transformants per viable cell at the time of plating.

## **Results and Discussion - I**

I Competition in the chemostat between E. coli strains with and without plasmid RP4

i) Plasmids RP4 and RP4-Δ in continuous culture - description of strains

ii) Maintenance of RP4 and RP4-Δ under conditions of phosphate or glucose limited growth

iii) Competition experiments between W3110 and W3110(RP4)

iv) Persistance of the R+ strain at low levels in the chemostat

v) Repeated competition with 'cycled' strains of W3110(RP4) and W3110

i) <u>Plasmids RP4 and RP4-Δl in continuous culture - description of strains</u>

The plasmid RP4 seems closely related to RP1; both plasmids share the same pattern of antibiotic resistance (kanamycin, ampicillin and tetracycline) and both belong to the same incompatibility group (Inc P) indicating similarity in their modes of replication. Recent reports (Burkarht et al., 1978; Burkarht et al., 1979) using the technique of partial denaturation mapping suggest that both plasmids also share similar physical features. Despite this similarity, each plasmid is thought to be capable of altering its phenotypic and physical state depending on the genetic background of its host (Causey and Brown, 1978).

The P group plasmids in general are of particular interest to molecular genetics as a result of their ability to replicate in a wide variety of Gram negative hosts, for this reason they have been proposed as possible vehicles for the movement of foreign DNA fragments between diverse Gram negative species (Meyer et al., 1977).

A naturally occuring deletion derivitive of plasmid RP4 has been isolated and characterised by Hedges et al. (1976). The deleted plasmid RP4-Δl lacks the ability to mediate conjugation with female E. coli and has lost one antibiotic resistance marker (kanamycin). RP4-Δl has been suggested as a vector for foreign DNA considered hazardous if equipped with the ability of self-transfer .

As described in a previous section, RP1 has been shown to be stably inherited in E. coli in the absence of selective pressure for at least

60 generations. Despite this observed stability in *E. coli*, RP1 has been shown to segregate from other bacterial species (Little, 1982). Moreover, upon long term storage at 37°C Klemperer *et al*. (1979) found loss of RP1 associated markers, the extent to which this observation reflected active degredation of the plasmid in starvation conditions was not determined.

Like many other large conjugative plasmids, RP1 and RP4 are maintained at low copy numbers within the cell. By a variety of techniques the absolute number of plasmids has been determined to be 1-3 per genome equivalent (Bukhari *et al*., 1977). With such a low number of plasmids per cell, the probability of a plasmid free cell arising would be high unless there existed a mechanism to correct for any imbalance in the distribution of daughter molecules into dividing cells.

In order to examine whether such a mechanism was present on these plasmids, RP4 was taken as the archetypal plasmid and subject to long term cultivation in the absence of selective pressure. In addition RP4-$\Delta$1 the transfer defective derivative of RP4 was also examined for its stability in continuous culture.

### ii) Maintenance of RP4 and RP4-$\Delta$1 under conditions of phosphate or glucose limited growth

Strain W3110 (RP4) was obtained from Dr. A. Atkinson (P.H.L.S., Porton Down) and its phenotype checked by growth on plates containing either ampicillin, tetracycline or kanamycin. Once satisfied with the strain's characteristics, W3110 (RP4) was grown in L-Broth supplemented with

Table 2.

Strain W3110(RP4)

(a)

| LIMITATION USED | DILUTION RATE (Hours) | LENGTH OF RUN (Generations) | MARKER LOSS | TRANSFER ABILITY AT END OF RUN |
|---|---|---|---|---|
| PHOSPHATE | 0·3 | 200 | - | + |
| | 0·1 | 150 | - | + |
| GLUCOSE | 0·3 | 50 | - | + |

(b) Strain C(RP4 $\Delta$ I)

| | | | | |
|---|---|---|---|---|
| PHOSPHATE | 0·3 | 54 | - | NA |
| GLUCOSE | 0·3 | 36 | - | NA |

NA - Not Applicable

ampicillin and used as an inoculum for a chemostat of 350 ml working volume. After a suitable batching up period the flow rate of incoming media (designed to limit growth by phosphate- or glucose-limitation) was set to a pre-calibrated value and the culture left to equilibrate for about 10 generations.

Samples of the resident population were removed at 5 or 10 generation intervals for periods up to 200 generations and plated for viable count. The percentage of population carrying plasmids was obtained by replica plating about 200 colonies in each case onto agar plates containing one of each of the three antibiotics. By this technique any fragmentation of the plasmid leading to loss of markers could be detected.

Table 2(a) shows the result of such an analysis for two different nutrient limitation (phosphate and glucose) each at two different dilution rates. No loss of markers was detected in up to 200 generations of nutrient-limited growth. Moreover, no transient loss of any antibiotic marker was noted and the ability of RP4 to self-transfer by conjugation was maintained at the end of each chemostat run. The last observation was examined by conjugation of W3110 (RP4) $Nal^s$ with E. coli J51 ($Nal^r$) and selection of exconjugants on plates containing ampicillin and naladixic acid.

Maintenance of RP4 for such long periods in the chemostat suggested that either the plasmid exhibited remarkable hereditary stability or that a constant loss and re-acquisition of the plasmid was occuring. The fact that RP4 was conjugatively competent could allow plasmid tranfer between E. coli cells within the chemostat. This was tested by repeating these experiments with the transfer defective RP4 derivative, RP4-Δ1 (Hedges et al., 1976). Table 2(b) shows that although the range of experiments done with RP4- 1 was less than with RP4, no loss of markers was ever observed. The hereditary stability of RP4 was thus shown to be maintained by RP4-Δ1 suggesting in both cases a 'tight' linkage between plasmid replication and cell division.

One possibility for the maintenance of 100% plasmid carrying cells could be that R- cells once they arose were less fit for chemostat growth than the RP4(+) isogen and were thus washed out of the chemostat and never detected. In order to allow for this possibility, enforced competition experiments were done.

iii) Competition experiments between W3110 and W3110 (RP4)

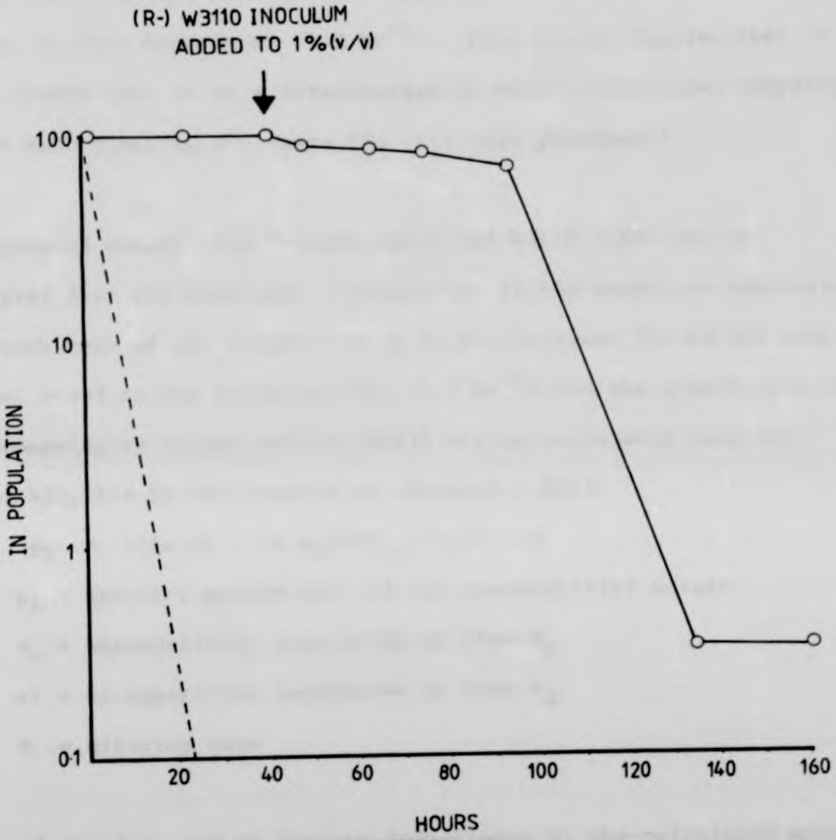
Competition experiments were performed by the addition of a small inoculum of the relevant plasmid free strain (~1% v/v) that had previously been grown in A.M.S. to mid-exponential phase, to a chemostat culture of the R+ strain running under phosphate limitation; following the results of Melling et al. (1977) only phosphate limitation was used as the limiting condition for competition between W3110 (RP4) and W3110. After the addition of the R- inoculum samples were removed and plated in the normal way.

Figure 2 shows the typical response of a phosphate limited culture of W3110 (RP4) challenged as described above. After a lag time of about 5 generations, presumably for adaption to the chemostat environment, the original R+ culture was rapidly replaced by the R- isogen.

Figure 2. Competition between strains with and without plasmid RP4.

A culture of E.coli W3110(RP4) growing at a dilution rate of $0{\cdot}3hrs^{-1}$ under phosphate limitation was challenged by a small inoculum of E.coli W3110 as described in the text.

The culture was sampled throughout the subsequent period of growth and the percentage of culture resistant to ampicillin, tetracycline and kanamycin is indicated (O——O) as is the theoretical washout rate for a non-growing organism at this dilution rate (------).


(R-) W3110 INOCULUM
ADDED TO 1%(v/v)
PERCENTAGE (RP4+) ORGANISMS IN POPULATION
100
10
1
0·1
20
40
60
80
100
120
140
160
HOURS

The kinetics of the takeover event were exponential and the rate of reduction of the R+ population closely resembles that of the theoretical washout rate (T.W.R.) of a non-growing organism at the dilution rate employed in this experiment (0.3 $hr^{-1}$). This result implies that it is the R+ strain that is at a disadvantage in mixed populations competing for the same limiting substrate (in this case phosphate).

The degree of competition between W3110 and W3110 (RP4) can be calculated from the kinetics of changeover in the chemostat population. The growth rate of the strain coming to predominance (R- W3110) must be at least equal to the dilution rate (0.3 $hr^{-1}$) and the growth rate of the uncompetitive strain (W3110 (RP4)) can be calculated from the washout kinetics by the formula of Jannasch (1967):

$$\mu_1 = [(\ln xt - \ln x_o)/(T_2 - T_1)] + D$$

where $\mu_1$ = specific growth rate of the uncompetitive strain

$x_o$ = uncompetitive population at time $T_1$

$xt$ = uncompetitive population at time $T_2$

and $D$ = dilution rate

Using values of $x_o$ and xt derived from figure 2, the calculated value of $\mu_1$ is 0.1 $hr^{-1}$ a reduction compared to the competing strain of 66%.

A similar result was obtained by Godwin and Slater (1979) and the calculated result for RP1 (Melling et al., 1977) is very similar. These values show that any R- cell arising within a population of R+ cells would be at a competitive advantage and would be sure to come to predominate the culture. Thus, conclusions about the remarkable efficiency of RP4 segregation into daughter cells are indeed justified, and the utility of long term cultivation of RP4 containing populations in the chemostat as a measure of plasmid stability has been demonstrated.

iv) <u>Persistence of the R+ strain at low levels in the chemostat</u>

After the enforced competition between W3110 (RP4) and W3110 in the chemostat had resulted in the percentage of R+ cells falling to about 0.1% of the total population, competition appeared to stop and the R+ strain was maintained at 0.01%-0.1% of the total population until the end of the experiment. A similar observation of persistence in chemostat culture has been reported in other competition situations (Dykhuizen, 1978; Melling et al., 1977; Zamenhof and Eichhorn, 1967; Godwin and Slater, 1979; Adams et al., 1979), all despite the prediction of chemostat theory that uncompetitive strains should be completely washed out of the chemostat (Powell, 1958).

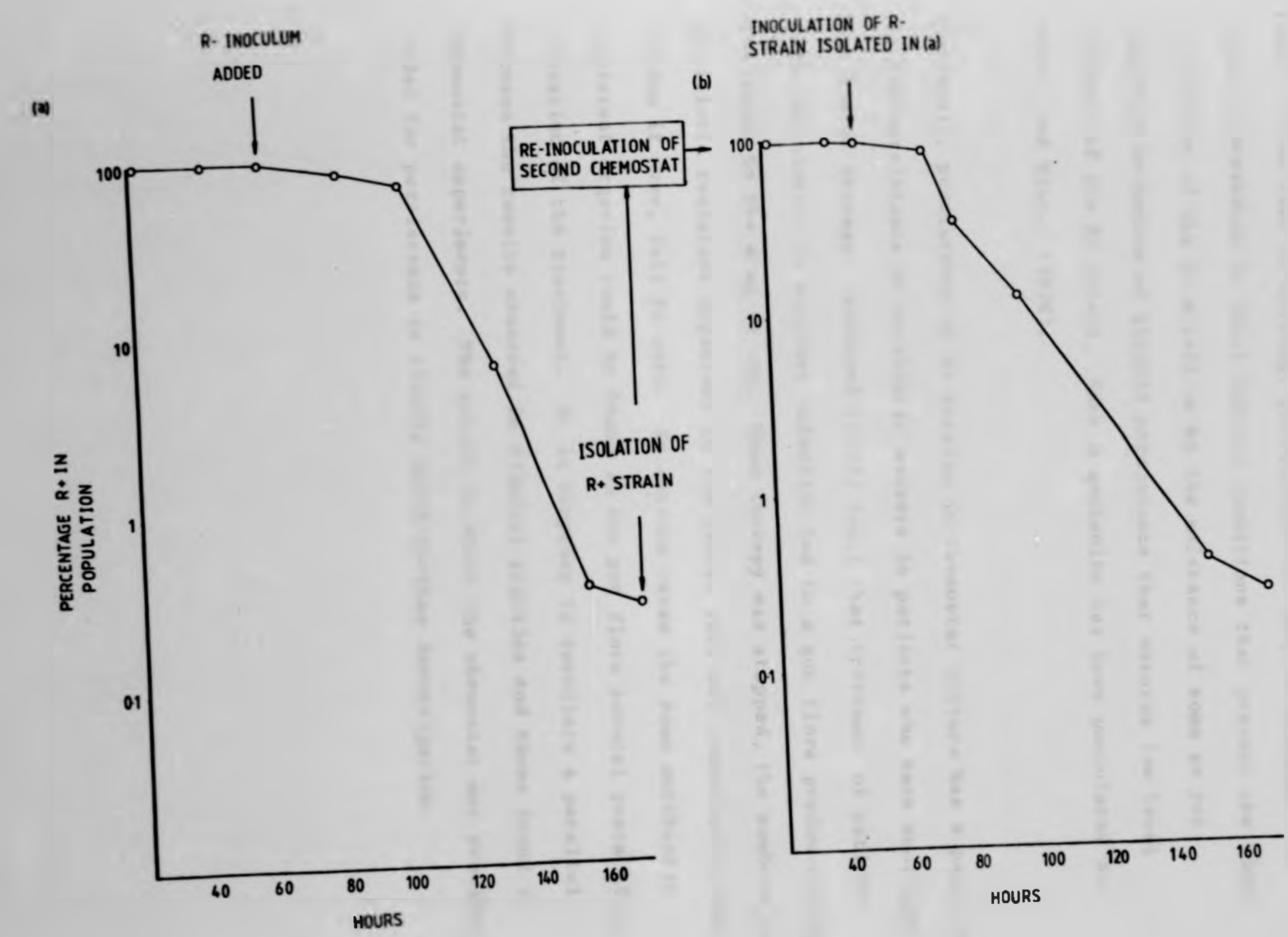
A possibility that would explain such persistence, especially in the case of plasmid bearing cells is that the presence of a plasmid alters bacterial cell wall layers and enables selective adhesion of R+ cells to the walls or baffles of the chemostat vessel. Some adhesion properties are known to reside on plasmids (e.g. Smith and Higgins, 1976) and so it was of interest to examine the wall population of a chemostat after a competition experiment to determine if indeed RP4 containing cells made up a high proportion of the adhering population. Wall growth was examined by rinsing a striped chemostat after a competition experiment several times in saline (0.9% w/v) and then swabbing the walls and baffles onto L-agar plates. Patching of the resultant colonies onto the three types of antibiotic plate allowed an estimation of the R+ and R- populations present. In all cases tested (several experiments) the relative proportions of R+ and R- cells present in the wall populations was the same as it was in free culture indicating a lack of specific adhesion due to the presence of RP4.

v) Repeated competition with 'cycled' strains of W3110 (RP4) and W3110

An alternative possibility for the persistance of R+ strains in mixed culture as a low level population could be mutation of those survivors to a higher growth rate such that peristance of these new 'fitter' strains was allowed. This possibility was tested in the following way: a culture of W3110 (RP4) maintained under phosphate limitation in the chemostat was subject to an enforced competition event in the normal way and allowed to washout to a residual (stable) level of 0.1%. At this point the R+ strain present in the chemostat was isolated on L-agar plates containing tetracycline and, after restreaking, used as inoculum for a fresh chemostat run under the same conditions as the first. After about 10 generations of limited growth this 'cycled' strain was challenged with an R- (W3110) inoculum isolated in a similar way from the original culture. If mutation had occurred in the persistant R+ population of the first chemostat and providing that the mutation was inherited during subculture, then competition in the second chemostat should be severely reduced or completely absent. Figure 3 shows the kinetics of washout for the R+ strain in each chemostat. Both rates of washout are similar and, in addition, the final R+ level in each case was the same at about 0.1% of the final population. The result implies

Figure 3. Repeated competition between strains with and without plasmid RP4 as a test of chemostat adaption as a cause of persistence.

A competition experiment similar to that shown in figure 2 was done using the strains E.coli W3110(RP4) and W3110. After competition had occured (3(a)), colonies representing the final R+ and R- populations were subcultured and used as competitive strains in a second chemostat run under identical conditions (fig.3(b)).



(a)
R- INOCULUM
ADDED
PERCENTAGE R+ IN POPULATION
100
10
1
01
40
60
80
100
120
140
160
HOURS
RE-INOCULATION OF SECOND CHEMOSTAT
ISOLATION OF R+ STRAIN
(b)
INOCULATION OF R- STRAIN ISOLATED IN (a)
100
10
1
01
40
60
80
100
120
140
160
HOURS

that, at the time of writing, persistence can only be explained as either a breakdown in ideal culture conditions that prevent the final elimination of the R+ strain or by the existance of some as yet undefined mechanism of plasmid persistence that ensures low level survival of the R+ strain. Such a mechanism has been postulated by Godwin and Slater (1979).

Curiously, persistence of R+ strains in chemostat culture has a parallel in the persistance of antibiotic markers in patients who have undergone antibiotic therapy. Richmond (1977) found that treatment of patients with antibiotics to suppress infection led to a gut flora predominantly resistant to the drug in use. When therapy was stopped, the numbers of antibiotic resistant organisms in the faeces fell off dramatically but, seldom if ever, fell to zero. In extreme cases the same antibiotic resistant organism could be found in the gut flora several years after cessation of the treatment. It is tempting to speculate a parallel between the results observed in clinical practice and those found in chemostat experiments. The extent to which the chemostat may provide a model for persistence is clearly worth further investigation.

## II Competition in the chemostat with other strain/plasmid combinations.

### i) Preview

### ii) Enforced competition with *E. coli* strains with and without pBR322

i) Preview

The results obtained in the preceeding chapter using E. coli W3110 (RP4) were similar to those published by Melling et al. (1977) with E. coli W3110 (RP1) and those reported by Wouters et al. (1978) using E. coli PC017 (R6).

This similarity in plasmid behaviour was not unexpected as all 3 plasmids share many biological features. It was however possible that the competition observed with these plasmid/host combinations was a property peculiar to them alone. Altered phosphate uptake for example associated with the presence of these large conjugative plasmids could have resulted in the severe disadvantage exhibited by the R+ strains studied under conditions of phosphate limitation. Indeed, RP1 is known to alter cell wall layers (Gilbert and Brown, 1978) and to affect the overall metabolism of its E. coli host (Klemperer et al., 1979). Other large plasmids such as R1 are also known to affect cell envelope structures (Nordstrom et al., 1977). Contrary to these fears and as described in a preceeding section limited competition has been reported for other plasmid/host combinations.

As a test therefore, of the generality of the conditions established in the preceeding chapter for the successful detection of R- strains should they arise in the chemostat, it was of interest to chose a different plasmid/host combination for further experiments. The plasmid chosen was plasmid BR322 (pBR322) (Bolivar et al., 1977) a plasmid whose biological properties of high copy number and non-conjugativeness were very different from those of either RP1 or RP4. This plasmid was of particular interest for several reasons; firstly, it was a member of a group of small multicopy plasmids whose replication was closely related to that of the much studied plasmid ColE1. Secondly, pBR322, although constructed from naturally occurring plasmids, was itself a novel synthetic product and it could not be isolated de novo from any naturally occuring E. coli strain, it was thus interesting to examine how such a novel construct might act in long term culture. Finally, at the time of writing, this plasmid is in wide use as a cloning vehicle for DNA inserts to be amplified and manipulated in E. coli. With proven techniques for the expression of some cloned DNA inserts in E. coli (e.g. Villa-Kamaroff et al., 1978; Itakura et al., 1977) and the likelyhood of commercial exploitation of beneficial products produced by such methods, it was of interest to examine the behaviour of pBR322 in chemostat culture, a technique of bacterial growth likely to be in use during the commercial production of genetically engineered products in E. coli.

ii) Enforced competition with E. coli strains with and without pBR322

In order to test the generality of phosphate-limited continuous culture for competition experiments, plasmid pBR322 was obtained in the host W5445 from Dr. S. D. Ehrlich (University of Paris) and this strain used without further maniplation as one of the resident R+ strains in chemostat culture. In addition pBR322 DNA was prepared from this strain and introduced into a variety of other laboratory E. coli strains by transformation. The strains so created were also used as residential R+ strains for enforced competition experiments in continuous culture.

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| *E.coli* HOST USED | DILUTION RATE OF EXPERIMENT | COMPETITION OBSERVED |
|---|---|---|
| W5445 | 0·25 | + |
| W3110 | 0·25 | + |
| HB101 | 0·25 | + |
| | 0·30 | + |
| C603 | 0·22 | + |

**Table 3.** Competition between *E.coli* strains with and without plasmid pBR322.
In all cases the culture was phosphate limited.

Competition was started after about 5 generations of limited growth by an inoculation of the relevant R- strain to a final concentration of about 1% (v/v). After inoculation the cultures were sampled regularly and examined for viable count and the percentage of plasmid containing organisms by the previously described procedure. In all cases the culture was phosphate limited and the dilution rate was set to a level that optimized the number of generations attained in a reasonable time period, in practice this was a value of around 0.3 $hr^{-1}$ (Td = 2.3 hr).

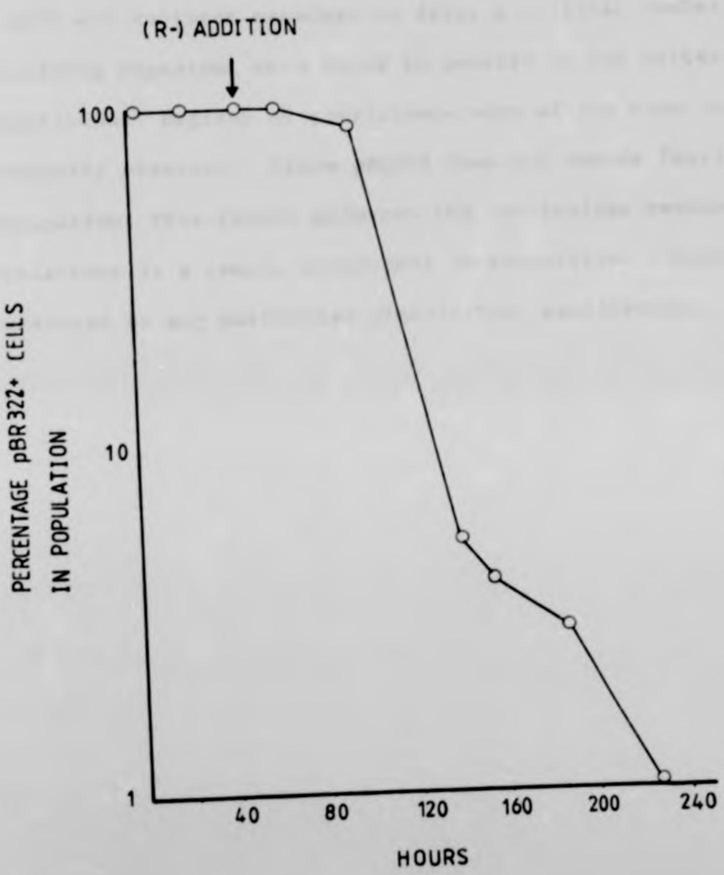
Table 3 shows the result of such an analysis, competition, that is the increase in the numbers of R- organisms from about 1% of the population to 99.9% of the population, was observed with every plasmid/host combination tested. Moreover, in each case competition was rapid and both antibiotic markers (ampicillin and tetracycline resistance) were lost at equal rates.

Figure 4 shows a typical result for the progress of competition after the introduction of the R- inoculum. In agreement with the previously examined competition between W3110 and W3110 (RP4), the rate of washout of the pBR322-containing strain was close to the theoretical washout rate for the dilution rate employed. Furthermore, the calculated decrease in $\mu max$ for the pBR322 carrying strain was, in each case about 60%, a figure very similar to that obtained for RP4 washout rates.

At first glance the loss of pBR322 organisms from the chemostat appears suspiciously rapid, however despite the small size of pBR322 (2.9 M. Dal) compared to RP4 ( 50 M.Dal), the number of plasmid copies per cell (20-30 for pBR322, 1-3 for RP1) (Bukhari et al., 1977) means that the

<u>Figure 4.</u> Competition between strains with and without pBR322.

An overnight culture of <u>E.coli</u> W5445(pBR322) was inoculated into a chemostat set to run under phosphate limited conditions at a dilution rate of 0·3 $hrs^{-I}$. After the establishment of a steady state (I0 - 20 generations), the culture was challenged with a small inoculum ( 0·I %, v/v) of the isogenic R- strain and the proportion of $Ap^R Tc^R$ organisms present in the population followed for the subsequent 80 generations.


(R-) ADDITION
100
10
1
PERCENTAGE pBR322+ CELLS
IN POPULATION
40
80
120
160
200
240
HOURS

total amount of extragenomic DNA is similar in strains carrying either plasmid. If the copy number of pBR322 were to fall there should be a concomitant decrease in the amount of competition observed.

As with all cultures examined to date, a residual number of pBR322 containing organisms were found to persist in the culture after competition. Degrees of persistence were of the same order as previously observed. Since pBR322 does not encode functions for conjugation, this result enforces the conclusions reached earlier that persistence is a common occurrence in competition situations and is not restricted to any particular plasmid/host combinations.

## III The Segregational stability of pBR322 and other ColE1 related replicons in continuous culture

i) Description of strains

ii) Natural segregation in chemostat culture

iii) Copy number changes during prolonged chemostat growth

iv) Complementation studies

**Table 4.**

| Plasmid | Derivation | Relevant phenotype | Source |
| --- | --- | --- | --- |
| pBR322 | Cloning vector | $Ap^R, Tc^R$. | S.D.Ehrlich(Bolivar _et al_.1977) |
| pMB9 | Cloning vector | $Ap^R, IeI^+$. | A.Atkinson(Boyer _et al_.1977) |
| pHVI4 | pCI94 inserted at _Hind III_ site of pBR322. | $Ap^R, Cm^R$. | S.D.Ehrlich(Ehrlich,1978) |
| pDSII09 | ColEI::TnI | $Ap^R, IeI^+$. | D.Sherratt(Dougan & Sherratt,1977) |
| pDS4IOI | ColK::TnI | $Ap^R, Ik^+$. | D.Sherratt(Warren & Sherratt,1978) |
| RPI | Natural isolate | $Ap^R, Km^R, Tc^R$. | M.Richmond(Grinsted et al.1972) |

i) Description of strains

The results described in the preceeding chapter show that, irrespective of the host strain employed, long term culture of strains carrying pBR322 should, at least under conditions of phosphate limitation lead to the detection (by virtue of their competitive advantage) of any R-strains that arise.

In the light of this finding experiments were embarked upon to examine the long term stability of pBR322 and, for comparison, several other small multicopy plasmids in continuous culture. Continuous culture is particularly suited to the examination of the stability of multicopy plasmids for, as a consequence of their high number of copies per cell the probability of plasmid free cells arising in the absence of any active segregation mechanism, is very low. For example, the probability of a daughter cell failing to inherit a plasmid with a copy number of 30 is only 1 in $10^{-9}$ per generation (from the binomial distribution). Clearly then the ability to run an experiment for many generations is a prerequisite to any study of multicopy plasmid stability.

A list of the plasmids used in this comparitive study is presented in Table 4. A detailed description of each plasmid may be obtained from the references given in Table 4 but in order to understand the significance of future results a short description of their biological properties and relatedness (if any) is appropriate here.

The plasmid pBR322 has already been described in some detail. It is the final product of a 'scramble and select' regime of vector construction. Very little space on the molecule is phenotypically silent; in addition to encoding resistance to ampicillin and tetracycline, the molecule replicates efficiently and maintains a copy number in rich media of about 25 plasmids per genome equivalent (Bukhari _et al._, 1977).

Plasmid pMB9 is one of the parental molecules used to generate pBR322. Its only phenotypic traits are resistance to tetracycline and immunity to the action of Colicin El. pMB9 is derived from the naturally occuring plasmid pMB1, itself a close relative of ColEl. pMB1 was reduced in size by deletion of several DNA fragments generated by the restriction enzyme Eco R1 (see Boyer _et al._, 1977) and marked with the tetracycline genes of an unrelated plasmid (pSC101) in order to create pMB9. pMB9 was subsequently marked with the amplicillin gene (from the transposon Tn1) and further size reduced by partial restriction to generate pBR322.

Plasmid pHV14 (Ehrlich, 1978) is a direct derivative of pBR322 that contains the additional genetic marker of resistance to chloramphenicol. It may be considered the same as pBR322 in terms of its biological behaviour in _E. coli_.

Plasmid pDS1109 is a direct descendant of the plasmid ColEl. The plasmid encodes resistance to ampicillin and immunity to the action of Colicin El. It does not itself produce Colicin El and is thus not self-selecting in conditions of mixed growth. pDS1109 may be regarded as highly related (but not identical to) PBR322 for, as judged by their inability to co-exist in the same cell, their modes of replication are very similar.

Figure 5 shows a simplified diagram of the relatedness of each of the plasmids so far described.

In addition to this small group of ColE1 related plasmids two other unrelated plasmids were included in this study as donors of possible factors involved in segregation.

RP1 has already been described and shown to exhibit stability in chemostat culture. Plasmid pDS4101 is a plasmid with biological properties similar to both pDS1109 and pBR322; it is maintained at high copy number and is resistant to ampicillin. However, pDS4101 is compatible with both pDS1109 and pBR322 indicating a sufficient difference in replication properties to allow both molecules to co-exist in the same cell. It has been shown (Warren and Sherratt, 1977) that pDS4101 can, when present in the same cell, supply some <u>trans</u>-acting functions that complement deficiencies in ColE1 derivitives that are unable to undergo mobilization via the conjugation apparatus supplied by a third plasmid. How many <u>trans</u>-acting functions can be complemented by pDS4101 is not known.

Armed with this small library of plasmids, experiments were done to investigate several aspects of their stability, viz:

1) Is each plasmid stably inherited in long term culture as for example RP1 and RP4 are?
2) If any plasmid is not stable then under what conditions do R-segregants arise?

Figure 5. A simplified scheme outlining the relatedness of each plasmid described in the text.

Step I - the tentative relationship between pMBI, ColEI and a postulated intermediate plasmid of unknown origin.

Step 2 - the transposition of the ampicillin transposon TnI from RP4 to ColEI.

Step 3 - size reduction of pMBI and ligation to the tetracycline resistance determinants of plasmid pSCIOI to yield plasmid pMB9.

Step 4 - transposition of the ampicillin transposon Tn3 onto pMB9 followed by subsequent rearrangement and size reduction leads to plasmid pBR322.

Step 5 - ligation of plasmid pCI94 to pBR322 to give plasmid pHVI4.

Key: ColEI$^{+}$- production of colicin EI, IcEI - immunity to colicin EI, EcoRI - production of the EcoRI restriction/modification enzymes, Ap$^{R}$- ampicillin resistance, Tc$^{R}$- tetracycline resistance, Cm$^{R}$- chloramphenicol resistance.

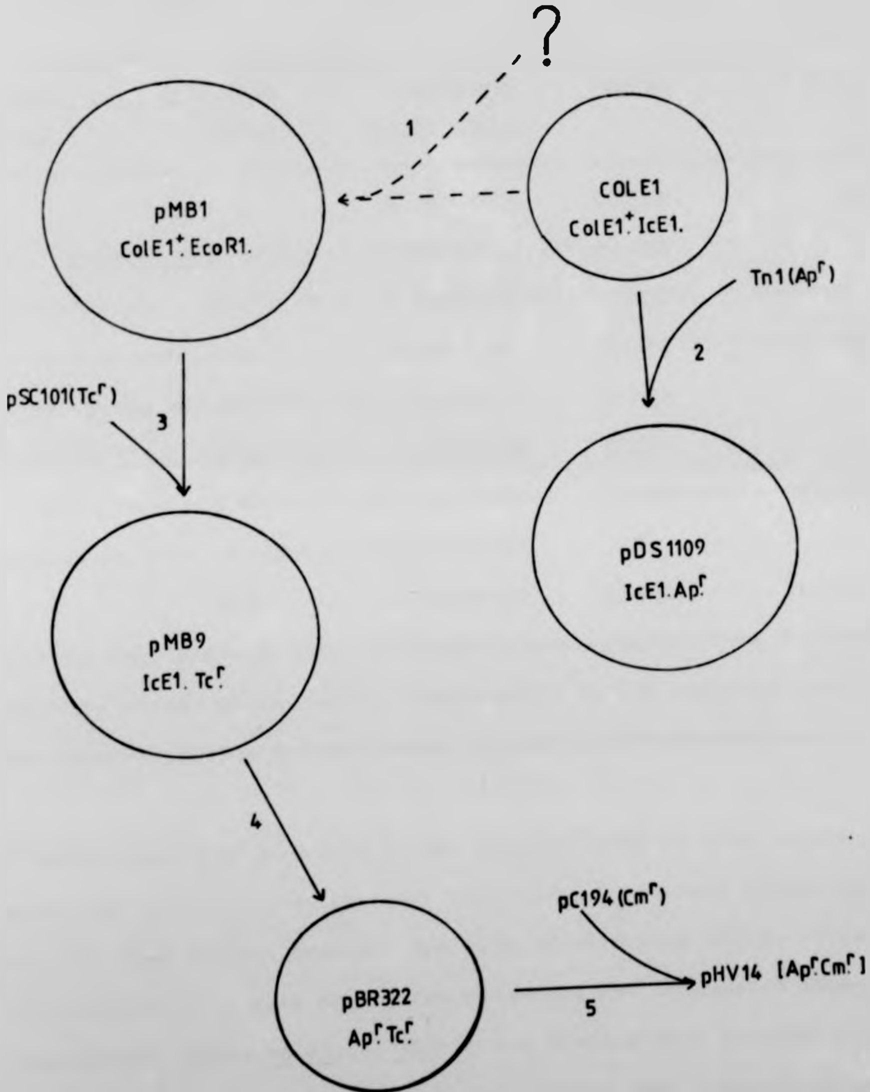

?
1
pMB1
ColE1⁺. EcoR1.
COL E1
ColE1⁺. IcE1.
Tn1 (Ap^r)
2
pSC101(Tc^r)
3
pDS1109
IcE1. Ap^r.
pMB9
IcE1. Tc^r.
4
pC194 (Cm^r)
pHV14 [Ap^r. Cm^r]
pBR322
Ap^r. Tc^r.
5

Table 5.

| PLASMID | HOST | NUTRIENT LIMITATION | DILUTION RATE | SEGREGANTS DETECTED |
|---|---|---|---|---|
| RPI | W3IIO | PHOSPHATE | 0·I | - |
| | W5445 | GLUCOSE | 0·2 | - |
| | W5445 | GLUCOSE | 0·3 | - |
| pBR322 | W5445 | PHOSPHATE | 0·2 | + |
| | W3IIO | GLUCOSE | 0·4 | + |
| | HBIOI | GLUCOSE | 0·4 | + |
| pDSII09 | W5445 | GLUCOSE | 0·2 | - |
| | " | PHOSPHATE | 0·2 | - |
| pDS4IOI | W5445 | GLUCOSE | 0·2 | - |
| pMB9 | W5445 | PHOSPHATE | 0·25 | + |
| | " | GLUCOSE | 0·2 | + |

3) What effect (if any) does long term culture have on plasmid replication and copy number?

4) Can any difficiency in segregation exhibited by these plasmids be complemented by the presence of a stable (and compatible) plasmid in the same cell?

ii) <u>Natural segregation in chemostat culture</u>

Initially the plasmids shown in Table 4 were screened in continuous culture for their ability to be maintained efficiently under conditions of phosphate and glucose limitation.

In most cases the dilution rate was 0.2 $hr^{-1}$, corresponding to a mean generation time of about 3 hr.

Strains were grown in antibiotic media immediately before inoculation into the chemostat to ensure only R+ cells in the original population. The results of this initial survey are shown in Table 5.

It would have been possible to use plasmid ColE1 in this survey as long as it was in a colicin-resistant background to prevent selection against plasmid free cells. However, for ease of selection and to avoid the possibility of colicin production affecting the outcome of these experiments (Adams <u>et</u> <u>al</u>., 1979) it was decided that pDS1109 should be the archetypal plasmid. As discussed earlier this plasmid no longer produces the cytotoxic protein Colicin E1.

These experiments consistently failed to detect any R- segregants of strains harbouring RP1, pDS4101 or pDS1109 even after 120 generations of nutrient limited growth. The type of competition from initially mixed culture reported earlier could however be shown for pDS1109 under conditions of phosphate limitation indicating that R1 segregants would have taken over the culture should they have arisen.

By contrast to the results obtained with pDS1109 in chemostat culture, natural R- segregants of strains harbouring either pMB9 or pBR322 were detected in the culture after approximately 30 generations. This observation was not dependent on any of the host genetic backgrounds tested. Plasmid-free segregants arose under both phosphate and glucose limitation, a surprising finding in view of the results obtained with RP4 (cf. Results I), but in agreement with the results of Adams *et al.* (1979) and Godwin and Slater (1979) both of whom observed competition under conditions of glucose limitation.

In agreement with all competition results to date a residual level of p BR322 or pMB9 containing cells was maintained at the end of competition.

The rate of washout of the R+ strain under either glucose or phosphate limitation was the same but, in each case markedly slower than in the enforced competition events described in Results Chapter II.

Figure 6 shows the progress of a typical culture of *E. coli* harbouring pBR322 and grown under phosphate limitation. The results are compared to those obtained by an enforced competition involving RP1 (6(a)) and those already described in Results II when a small R- inoculum was added

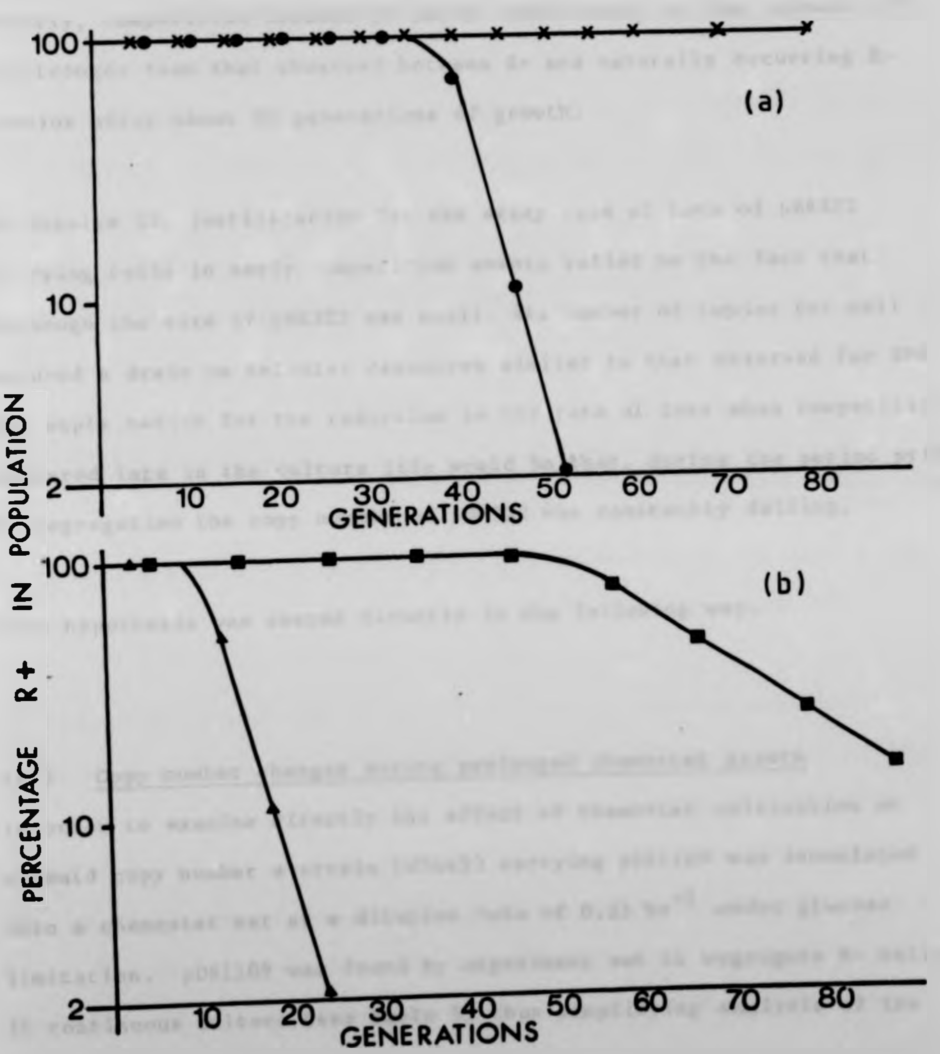
Figure 6. Relative washout kinetics of pBR322- and RPI- containing cells in phosphate limited chemostats. The dilution rate was set at 0·25 $hrs^{-I}$.

(a) X——X, RPI-containing cells alone.

●——●, RPI-containing cells after the addition of a small (~0·I %) inoculum of isogenic RPI-free cells to the culture at about generation 30.

(b) ■——■, pBR322-containing cells in the absence of a plasmid free inoculum.

▲——▲, pBR322-containing cells after the addition of a small inoculum (~0·I %) of isogenic pBR322-free cells at about generation 5.



PERCENTAGE R+ IN POPULATION
(a)
100
10
2
10
20
30
40
50
60
70
80
GENERATIONS
(b)
100
10
2
10
20
30
40
50
60
70
80
GENERATIONS

to the culture at generation five (6(b)).

Clearly, competition between R+ and R- cells early in the culture life is stronger than that observed between R+ and naturally occurring R- strains after about 30 generations of growth.

In Results II, justification for the steep rate of loss of pBR322 carrying cells in early competition events relied on the fact that although the size of pBR322 was small, its number of copies per cell ensured a drain on cellular resources similar to that observed for RP4. One expla nation for the reduction in the rate of loss when competition occurred late in the culture life would be that, during the period prior to segregation the copy number of pBR322 was constantly falling.

This hypothesis was tested directly in the following way.

iii) Copy number changes during prolonged chemostat growth

In order to examine directly the effect of chemostat cultivation on plasmid copy number a strain (W5445) carrying pDS1109 was inoculated into a chemostat set at a dilution rate of 0.25 $hr^{-1}$ under glucose limitation. pDS1109 was found by experiment not to segregate R- cells in continuous culture (see Table 5) thus simplifying analysis of the data.

During the culture run, cell samples were removed for analysis of viable count and percentage of plasmid carrying cells as normal. In addition, each sample was analysed for plasmid copy number by two independent methods; the amount of plasmid DNA in a fixed volume culture was determined by the separation and quantification of plasmid DNA from a crude cell lysate (Twigg and Sherratt, 1980). Because the culture was maintained in steady state equal volumes of culture can be directly compared in this way. Separately, each sample of culture was plated on L-agar plates containing varying amounts of ampicillin. By this method the minimum inhibitory concentration (m.i.c.) of ampicillin was determined for each cell sample. Because the plasmid encoded - β-lactamase is constitutively expressed the amount of enzyme produced (and so the value of the m.i.c.) is dependent on the copy number (Uhlin and Nordstrom, 1975).

Figure 7 shows the results of this analysis; culture samples taken periodically were directly analysed for plasmid content on agarose gels (a) and separately the m.i.c. for ampicillin was determined (b).

The amount of plasmid DNA in each gel track was determined from microdensitometry of the photographic negative and is compared in figure 8 to the value obtained by the m.i.c. values. During the period of growth examined (82 generations) the copy number of pDS1109 fell five fold as determined by gel analysis and four fold as determined by the m.i.c. for ampicillin. The kinetics of reduction in copy number are approximately linear and are consistant with the interpretation of a gradual decrease in the number of plasmid copies per cell as opposed to the successive establishment of mutants with progressively lower copy numbers. Moreover, after a single cycle of growth on plates containing ampicillin the m.i.c. value of pDS1109 containing cultures returned to its original value (not shown).

Figure 7. Copy number analysis of pDSII09 during glucose limited continuous culture.

(a) Direct visualization of plasmid content was done as described in the text. Equal amounts of chromosomal DNA (equivalent to cell mass) are present in each sample.

(b) Samples removed for plasmid analysis in (a) were plated for viable count on L-agar plates containing varying concentrations of ampicillin. After overnight incubation the concentration of ampicillin causing a 50 % reduction in plating efficiency was recorded. The results are the average of duplicate experiments.

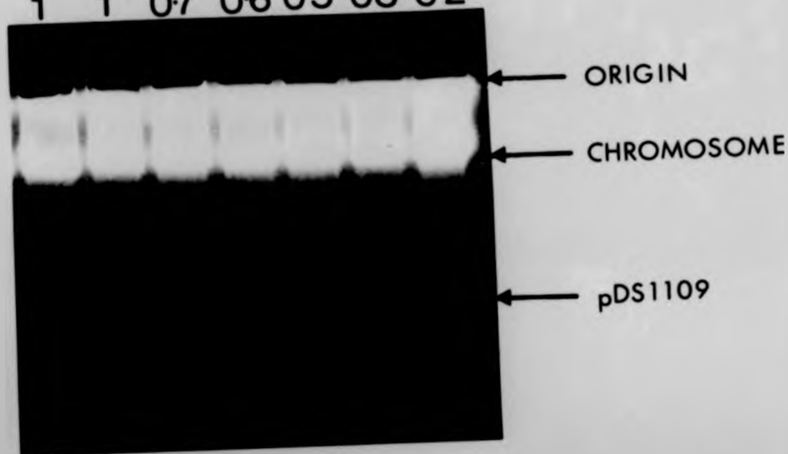
(a)

GENERATIONS

9 12 21 33 39 70 82

RELATIVE PLASMID CONTENT

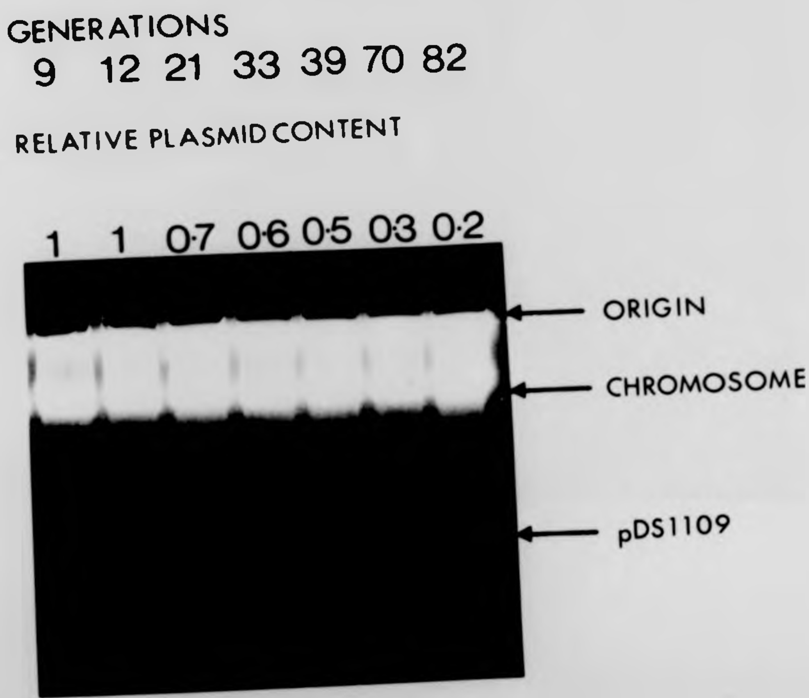
1 1 0·7 0·6 0·5 0·3 0·2




(b)

| GENERATIONS ELAPSED | M.I.C. FOR AMPICILLIN (µg/ml) |
|---|---|
| 9 | 3200 |
| 12 | 3000 |
| 21 | 2100 |
| 33 | 1800 |
| 39 | 1100 |
| 70 | 950 |
| 82 | 800 |

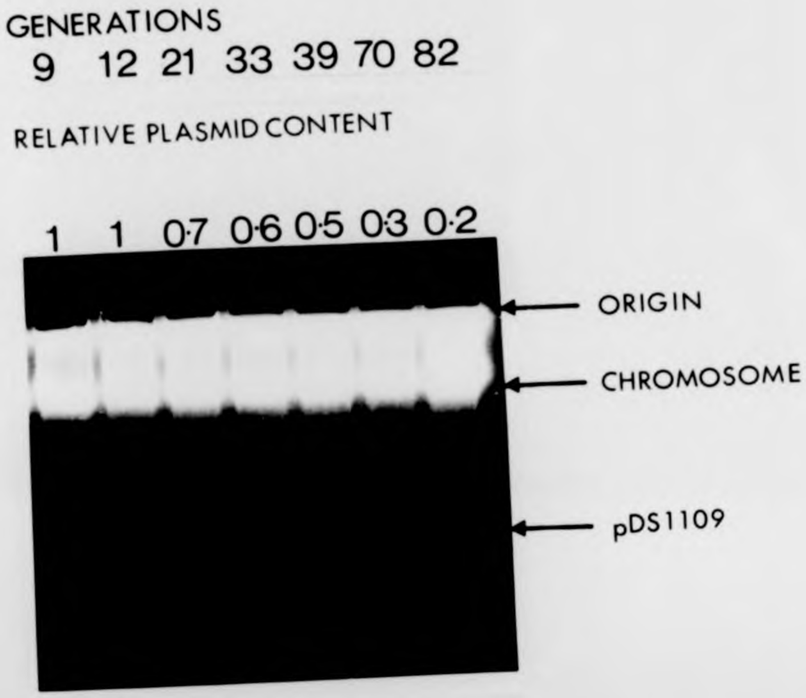
(a)




(b)

| GENERATIONS ELAPSED | M.I.C. FOR AMPICILLIN (µg/ml) |
|---|---|
| 9 | 3200 |
| 12 | 3000 |
| 21 | 2100 |
| 33 | 1800 |
| 39 | 1100 |
| 70 | 950 |
| 82 | 800 |

(a)




(b)

| GENERATIONS ELAPSED | M.I.C. FOR AMPICILLIN (μg/ml) |
|---|---|
| 9 | 3200 |
| 12 | 3000 |
| 21 | 2100 |
| 33 | 1800 |
| 39 | 1100 |
| 70 | 950 |
| 82 | 800 |

Figure 8. The kinetics of copy number drop during chemostat culture of E.coli W5445 pDSII09.

Points on the graph were taken from those presented in figure 7.

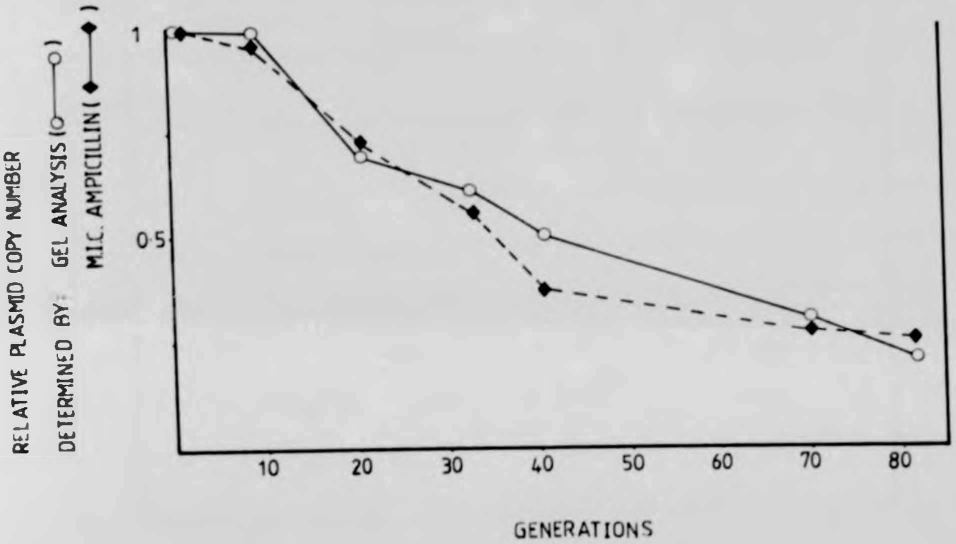

RELATIVE PLASMID COPY NUMBER
DETERMINED BY: GEL ANALYSIS (o——o)
M.I.C. AMPICILLIN (◆——◆)
1
0·5
10
20
30
40
50
60
70
80
GENERATIONS

TABLE 6

| PLASMID COPY NUMBER | PROBOBILITY OF R- CELL ARISING IN ABSENCE OF PARTITION MECHANISM |
|---|---|
| I | I |
| 2 | 0·5 |
| 4 | $I{\cdot}25 \times IO^{-I}$ |
| IO | $I{\cdot}9 \times IO^{-3}$ |
| 20 | $I{\cdot}7 \times IO^{-6}$ |
| 30 | $I{\cdot}7 \times IO^{-9}$ |
| 40 | $I{\cdot}7 \times IO^{-12}$ |

Assuming an initial value for the copy number of pDS1109 as about 15-20 copies per genome equivalent the calculated copy number at the end of the culture period was 3-4 plasmids per cell. Despite this no R- segregants were detected in the culture suggesting an efficient mechanism of segregation into daughter cells at division. The plasmid pBR322, despite its close relationship with pDS1109 is evidently not as efficient in segregation, for R- segregants are detected in continuous culture.

If no active mechanism of plasmid segregation exists, the probability of establishing plasmid-free cells depends entirely on the plasmid copy number and is described mathematically by the binomial distribution where:

$$P = 2\,(0.5)^{c}$$

where P is the probability of a daughter cell not receiving a plasmid and c is the copy number of that plasmid.

A table of representative values for P and c is shown in table 6. By reference to results obtained with pDS1109, the copy number of pBR322 would fall by about 50% during the period of 30-40 generations of growth prior to the detection of segregants in cultures of E. coli (pBR322). Referral to Table 6 shows that the probability of a segregant arising after a 50% reduction in copy number increases by a factor of between $10^3$ and $10^6$ depending on the initial copy number of pBR322 (20-30, Bukhari et al., 1977).

The decrease in copy number during the period of growth leading up to segregation of pBR322-free cells can thus explain not only the reduction in the degree of competition observed when compared to enforced competition early in the culture life (cf. Fig. 6(b), but also the lag period itself; efficient segregation of R+ cells during this period is explained simply by the random distribution of high numbers of plasmid copies between dividing cells.

iv) Complementation studies

The results of the preceeding chapter strongly suggest the presence on pDS1109 and presumably other 'natural' ColE1 related plasmids, of an element or elements responsible for the active partitioning of plasmid molecules into newly formed daughter cells.

Plasmid pMB9 and pBR322 fail to exhibit the same degree of hereditary stability and are thus considered to have lost the function necessary for efficient partitioning.

The constructions of pMB9 and pBR322 have been briefly discussed in an earlier section and it is possible that it was during these constructions that the partition (par) element equivalent to that of pDS1109 was lost or deleteriously altered. It was therefore of interest to examine the known biological properties of both pDS1109 and pBR322 with a view to ascribing the loss of 'par' action to the loss of any known loci.

By comparison with pDS1109 both pMB9 and pBR322 are known not to form relaxation complexes, the DNA/protein complex described briefly in an earlier section as a complex involved in the conjugal transfer of ColE1 like plasmids (Warren *et al.*, 1978) but also known to affect DNA replication (Collins *et al.*, 1978).

The inability of pBR322 to form a relaxation complex can be complemented *in trans* by the presence of the plasmid ColK or one of its ampicillin marked derivitives (e.g. pDS4101) (see Warren and Sherratt, 1977). In order to ascertain wether or not the relaxation complex was involved in partition, strains were constructed that contained both pBR322 and pDS4101 (ColK).

In a separate study strains carrying both pBR322 and RP1 were constructed in order to examine the possibility of plasmid RP1 providing partition functions directly *in trans*. Both RP1 and pDS4101 can be stably maintained in the same cell as pBR322 and both are stably inherited in continuous culture (Table 5).

Strains were constructed as follows: pDS4101, a ColK:Tn 1 plasmid was obtained from Dr. D . Sherratt (University of Glasgow) and plasmid DNA prepared as described (Materials and Methods). *E. coli* W5445 was transformed with pDS4101 DNA and selection of transformants made on ampicillin agar plates. The strain so created *E. coli* W5445 (pDS4101) was made competent and transformed with pBR322 DNA; selection was made for tetracycline resistance. The final strain carrying both pDS4101 and pBR322 was maintained on tetracycline plates prior to inoculation of a chemostat.

A strain carrying both RP1 and a pBR322 derivitive (pHV14) was constructed in a similar fashion. pBR322 shares the ampicillin and tetracycline resistance markers of RP1 and so segregants could not be detected. pHV14, a chloramphenicol resistant derivative of pBR322 was therefore used in place of pBR322 and the percentage of $Cm^r$ organisms was determined throughout the chemostat run.

Chemostats set to limit growth with low levels of phosphate were inoculated with each of these strains, the dilution rate was set at 0.25 $hr^{-1}$ and the cultures were sampled for organisms resistant to ampicillin and tetracycline (pDS4101 + pBR322) or kanamycin and chloramphenicol (RP1 + pBR322) in the normal way.

Figure 9 shows the resultant pattern of marker loss exhibited by each culture. Tetracycline sensitive cells were detected in the culture of E. coli W5445 (pDS4101) (pBR322) after about 40 generations of growth and came to predominate the culture with kinetics similar to those observed when pBR322 alone was present in W5445 (fig. 6(a)).

Similarly, chloramphenicol sensitive organisms were detected in the second culture [E. coli W5445 (RP1) (pHV14)] after a lag period of about 40 generations and came to predominate the culture with characteristic kinetics (Fig. 9(b)).

In both cultures the markers associated with the plasmid supplied in trans ($Ap^r$ for pDS4101, $Km^r$ for RP1) were maintained in 100% of the population confirming earlier findings (Table 5).

In addition to the monitoring of population markers during these experiments, in the case of cultures of W5445 (pDS4101) (pBR322)

Figure 9. Complementation studies on the segregation of pBR322.

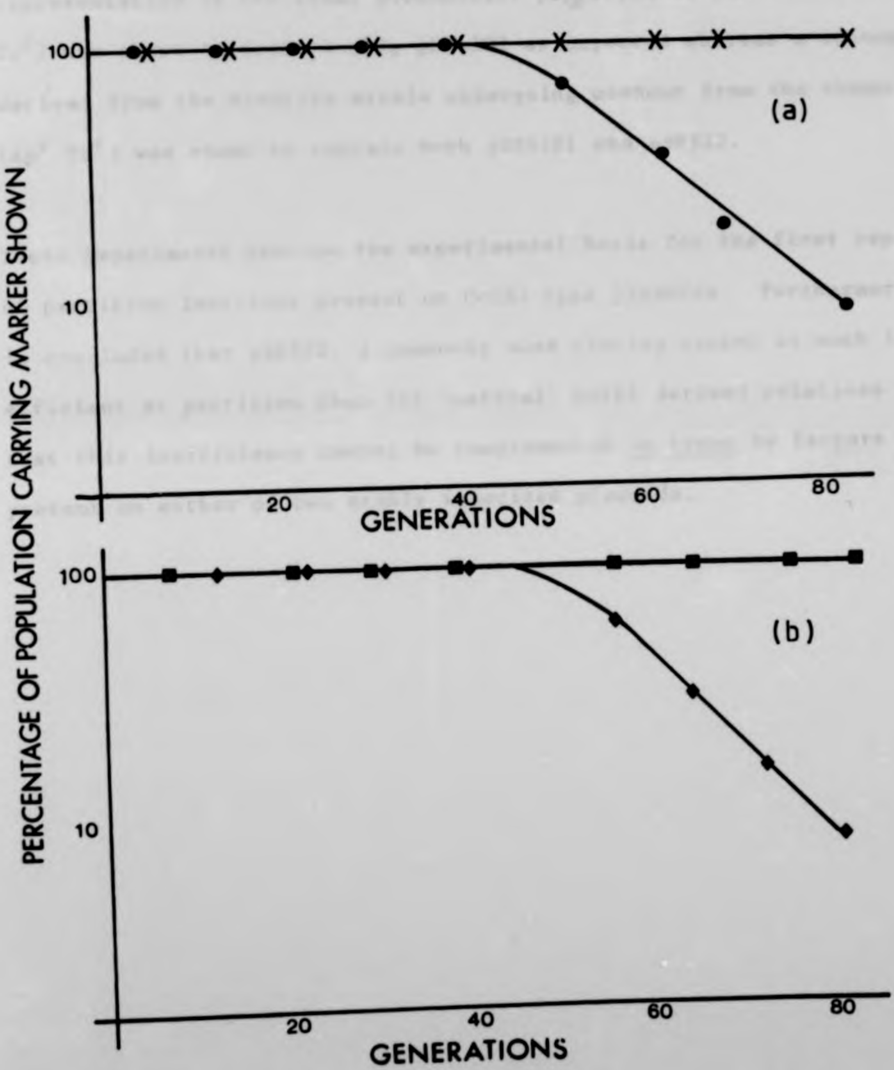
The kinetics of loss of pBR322 associated markers from E.coli W5445 containing either pDS4101 (a) or RP1 (b) are shown for phosphate limited cultures run at a dilution rate of $0 \cdot 25$ $hrs^{-1}$.

Symbols are : (a) X——X, $Ap^R$ of pDS4101.

●——●, $Tc^R$ of pBR322.

(b) ■——■, $Km^R$ of RP1.

◆——◆, $Cm^R$ of pHV14 ($Cm^R$ - pBR322).


PERCENTAGE OF POPULATION CARRYING MARKER SHOWN
100
10
(a)
20
40
60
80
GENERATIONS
100
10
(b)
20
40
60
80
GENERATIONS

colonies representing each of the final populations present in the chemostat at the end of the experiment were lysed and analysed for their plasmid content. Figure 10 shows the result of such an analysis. A representative of the final predominant population in the chemostat ($Ap^r$ $Tc^s$) was shown to contain only pDS4101 as expected whereas a colony derived from the minority strain undergoing washout from the chemostat ($Ap^r$ $Tc^r$) was shown to contain both pDS4101 and pBR322.

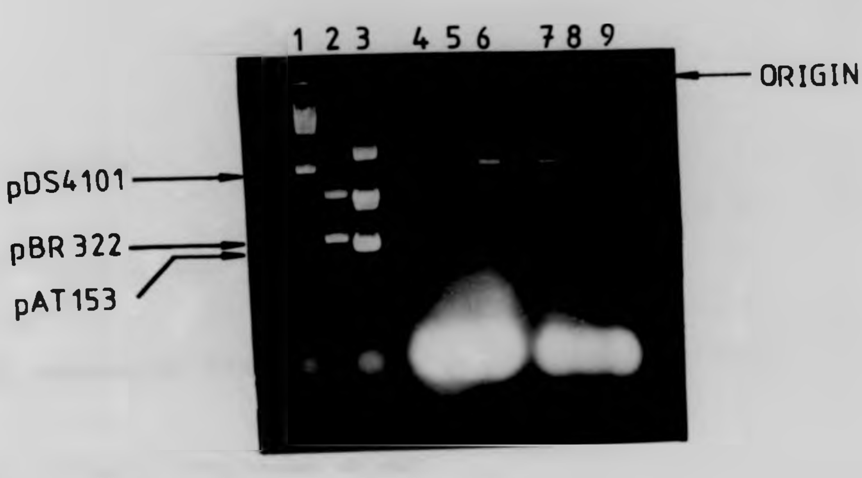
These experiments provide the experimental basis for the first reports of partition functions present on ColE1 type plasmids. Furthermore, it is concluded that pBR322, a commonly used cloning vector is much less efficient at partition than its 'natural' ColE1 derived relatives and that this inefficiency cannot be complemented _in trans_ by factors present on either of two stably inherited plasmids.

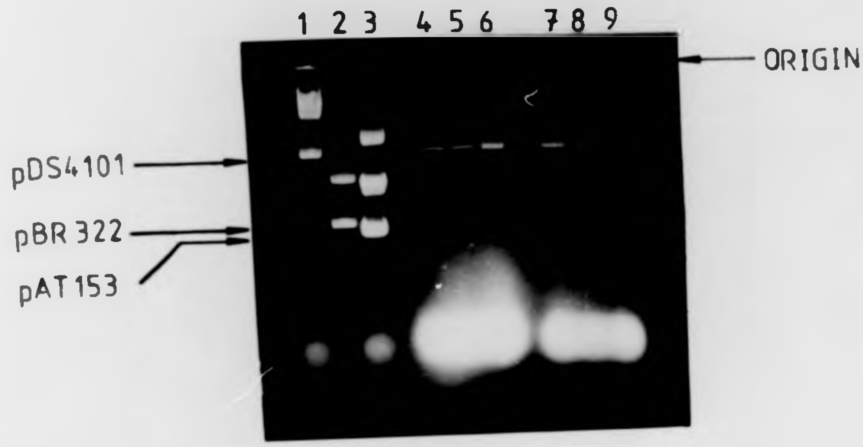
Figure IO. An analysis of the final populations present in the chemostat after loss of the $Tc^R$ marker during growth of W5445(pDS4IOI)(pBR322) (cf.fig.9a).

Tracks: I - 3 Marker plasmids as shown.

4 - 6 Three independent colonies from the predominant final population ($Ap^R$ $Tc^S$).

7 - 9 Three independent colonies representing the minority final population ($Ap^R Tc^R$).


1 2 3 4 5 6 7 8 9
ORIGIN
pDS4101
pBR 322
pAT153


1 2 3 4 5 6 7 8 9
ORIGIN
pDS4101
pBR322
pAT153

# IV Stabilization of the defective partitioning exhibited by pBR322

i) Preview

ii) The $\lambda$ rex function

iii) Cloning the $\lambda$ rex gene

iv) Chemostat cultivation of p$\lambda$12

v) Cloning of partition functions from pDS1109 and pPM31

vi) Cloning strategy from pDS1109

vii) Cloning strategy from pPM31

viii) The heriditary stability of plasmids pIJH7, pIJH1 and pIJ005

i) <u>Preview</u>

By the use of continuous culture it has been possible to distinguish a difference in the stability of populations of pDS1109, a direct descendant of ColE1 and a closely related plasmid pBR322.

R- segregants of the latter plasmid were detected in populations after a drop in copy number that was also exhibited by pDS1109. Despite the low copy number of pDS1109 after several generations of nutrient-limited growth, no segregants were detected within a population grown for more than 120 generations.

This observation suggested that pDS1109 encodes a function that is directly responsible for the efficient segregation of daughter molecules into newly divided cells. Since pBR322 is evidently deficient in this efficient partitioning mechanism, its stability might be increased by cloning the necessary segregation element from pDS1109 into pBR322. The segregational stability of recombinants so formed could then be tested by their cultivation in the chemostat under previously described conditions that allow the detection of R- segregants. Not only would such an experimental approach allow the definition of the region of DNA involved in partition but also, it would allow the expansion of such experiments to test other forms of stabilization. For example, the incorporation of a gene beneficial to the growth of the host may prevent competition by the continual selection for plasmid carrying cells.

In this chapter new plasmids are described that have been generated by the *in vitro* ligation of DNA fragments bearing complementary cohesive ends to form novel plasmids and whose stablity has been examined in continuous culture. In all the experiments described, the recipient plasmid for cloning was pBR322. The 'donor' DNA for each of the experiments described came from a variety of sources. Donor sequences for two experiments were derived from stably inherited replicons, and, in each case the cloned DNA fragment was shown to stabilize pBR322 in continuous culture. In a separate construction, a fragment of bacteriophage $\lambda$ DNA known to influence glucose metabolism was cloned into pBR322 and the recombinant grown under glucose limitation in the chemostat. Despite its successful expression the cloned fragment of $\lambda$ DNA failed to enhance the stability of pBR322-derived replicons within a population.

ii) The $\lambda$ rex function

A system that shares much with the experiments described in this thesis is that of the competitive growth of two *E. coli* strains that are isogenic apart from the carriage by one of them of an integrated prophage.

Although a lysogen requires little in terms of additional cellular energy for its maintenence (Echols, 1971), the amount of energy taken up by additional replication, transcription and translation must be greater than that required by the non-lysogenic counterpart.

It is not surprising then, that under conditions of anaerobic glucose limitation (energy limitation) non-lysogenic strains of *E. coli* outcompete lysogens in the chemostat in a similar manner to that observed between R- and R+ strains of *E. coli* (Lin *et al.*, 1977).

Contrary to expectations however, this observation is reversed if the two strains (lysogen and nonlysogen) are cultured together under aerobic glucose limitation (Edlin *et al.*, 1975; Lin *et al.*, 1977).

The ability of lysogens to outcompete non-lysogens under glucose limitation in the chemostat has been shown to extend to other lysogen/non-lysogen pairs (Edlin *et al.*, 1977) and, in the case of lambda lysogens the biological basis of this competetive advantage has been mapped to the $\lambda$ *rex* gene (Lin *et al.*, 1977). Lysogens constructed that are *rex* do not exhibit the competitive advantage of the wild type lysogen. The functional basis of the *rex* gene involvement is poorly understood but sensitive indications of oxidative metabolism have indicated that, during aerobic growth on glucose as sole carbon substrate lysogens can attain a higher efficiency of glucose utilization than non-lysogens. Since the manifestation of the *rex* phenotype (the prevention of infection by rII mutants of T4) involves changes in the cell wall layers, it has been suggested that, as an inadvertent consequence of such changes, glucose uptake by the sugar transport machinery present in the cell envelope layers is also, and beneficially altered (discussed in Edlin *et al.*, 1977).

In view of these published findings experiments were done to clone the *rex* gene from bacteriophage $\lambda$ onto plasmid pBR322. The stability of such a recombinant could be tested in continuous culture under glucose limitation.

The hypothesis under test in these experiments was the following:

Could the rex gene product present on the recombinant confer a competitive advantage on R+ cells in a similar manner to that conferred on $\lambda$ lysogens in the chemostat?

iii) Cloning the $\lambda$ rex gene

Bacteriophage $\lambda$ DNA has been characterized by many groups and the alignment of physical and biological maps used for these experiments is shown in Figure 11. For ease of experimental procedure and from the position of rex shown in Figure 11, $\lambda$ DNA was digested with enzymes that would leave the rex gene intact and would allow selection of recombinants by insertional inactivation of a resident gene on the vector plasmid (pBR322). To this end a library of $\lambda$ fragments inserted into pBR322 was constructed using the enzymes Bam H1 and Hind III, both of which allow the detection of recombinants by the insertional inactivation of tetracycline resistance normally expressed by pBR322.

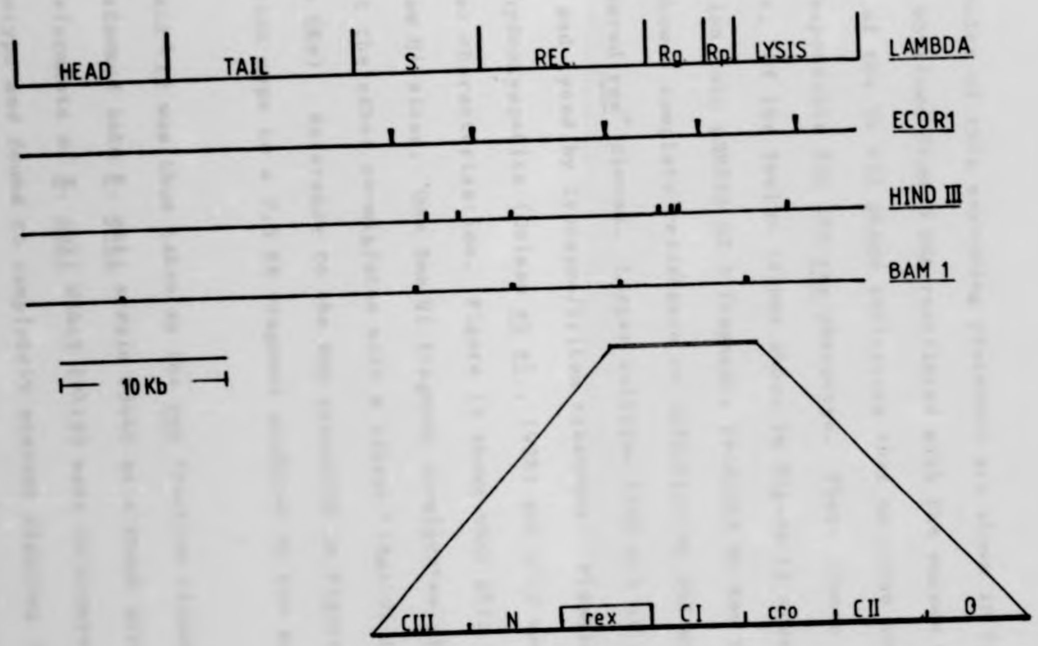
After ligation of suitably digested $\lambda$ DNA and pBR322 DNA followed by transformation of the ligated DNA into E. coli, about 500 ampicillin-resistant transformants were patched onto both ampicillin and tetracycline agar plates. A selection of $Ap^r$ $Tc^s$ clones were then grown in 5 ml batches and their plasmid content examined on agarose gels after rapid plasmid purification (see Materials and Methods). In addition, each recombinant was grown in 1 ml aliquots and the culture used as the recipient strain for infection by a suitable diluted stock of the T4 rII

Figure II. A biological map of bacteriophage lambda aligned with the sites for restriction enzymes EcoRI, HindIII and BamHI. The region encompassing the rex locus has been expanded for clarity.

Abbreviations used: Rg - Regulation.

Rp - Replication.

S - Region of relative genetic silence.

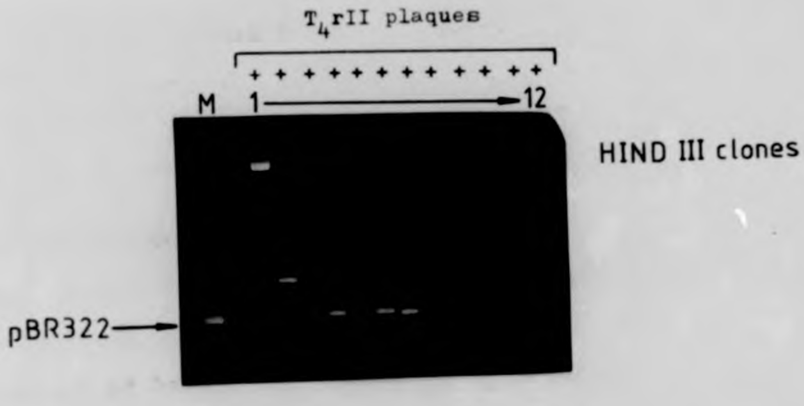

LAMBDA
HEAD
TAIL
S
REC.
Rg
Rp
LYSIS
ECOR1
HIND III
BAM 1
10 Kb
CIII
N
rex
CI
cro
CII
O

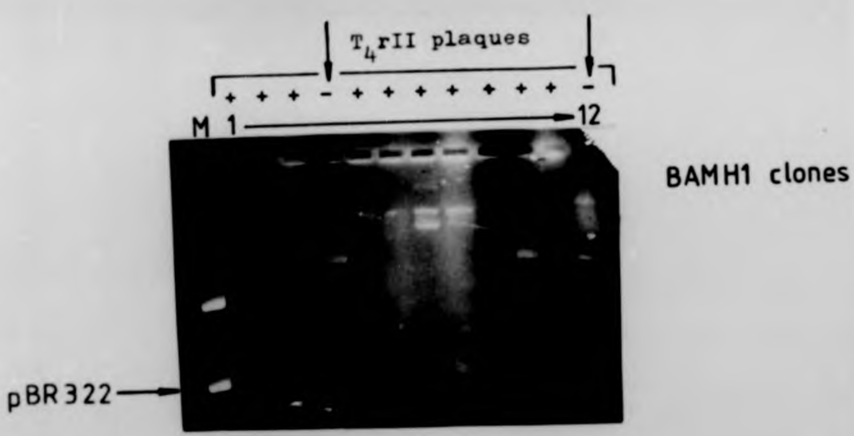
phage H88 (kindly supplied by Professor D. Ritchie, University of Liverpool). After overnight incubation each recombinant was scored for its ability to plaque H88.

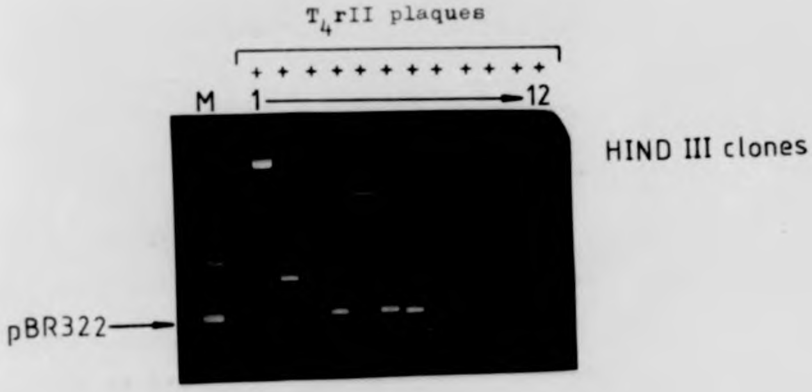
The results of this screening procedure are shown in Figure 12. All the clones obtained from λ DNA restricted with the enzyme Hind III supported growth of the T4 rII phage indicating that no clone contained the intact gene responsible for the rex phenotype. These clones were not analysed further. Of the twelve clones shown in Figure 12 constructed by the insertion into pBR322 of λ fragments produced by Bam HI, two, pλ4 and pλ12 showed complete resistance to infection by H88 and as such were considered $rex^+$ clones. Larger cultures (100 ml) of pλ4 and pλ12 were grown and lysed by lysozyme/triton treatment. Plasmid DNA was purified from hydroxyapatite (Colman et al., 1978) and pλ12 was chosen for further characterisation. Figure 13 shows that pλ12 DNA contains only two Bam H1 sites. One Bam H1 fragment co-migrates with linear pBR322 whilst the other co-migrates with a linear λ-Bam H1 fragment of 7.3 Kilo bases (Kb). Reference to the map presented in Figure 11 shows the λ rex function maps to a 7.3 Kb fragment produced by the enzyme Bam H1.

Plasmid λ12 was thus taken as the rex fraction cloned in pBR322 and was transformed into E. coli strain W5445 as a stock strain. Six transformants of E. coli W5445 (Pλ12) were re-assayed for the rex phenotype and found to completely prevent plaquing of phage H88. The rex gene was thus transferrable by transformation (plasmid borne) and stably expressed in E. coli.

**Figure I2.** Analysis of $Ap^RTc^S$ clones produced by insertion of restriction fragments of the lambda genome into pBR322 at either the HindIII or BamHI site. In each case (BamHI and HindIII) plasmid DNA from I2 independent clones was compared to that of marker pBR322 (Track M in each case). In addition and indicated in the panel above each gel, all clones were scored for their ability to support replication of the $T_4$rII mutant H88. Two clones are indicated (pλ(Bam)4 and pλ(Bam)I2) that both contained plasmids larger than pBR322 and failed to plaque phage H88.


T4rII plaques
+ + + + + + + + + + + +
M 1 12
HIND III clones
pBR322


T4rII plaques
+ + + - + + + + + + + -
M 1 12
BAMH1 clones
pBR322


T4rII plaques
+ + + + + + + + + + + +
M 1 12
HIND III clones
pBR322

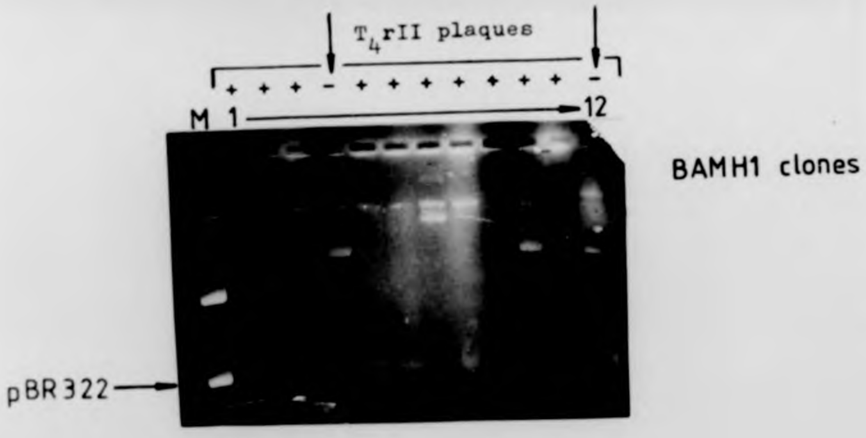
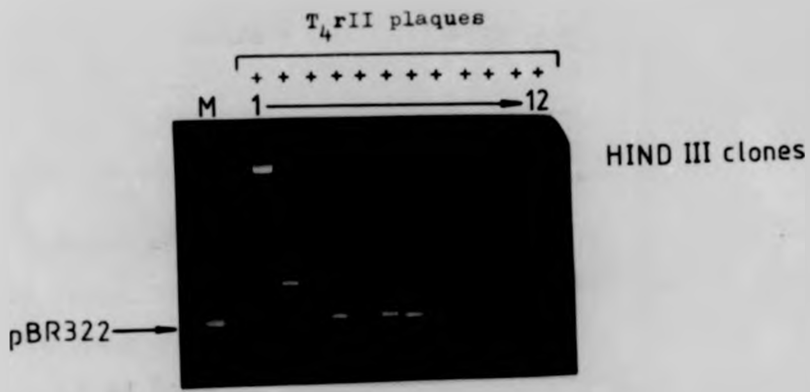
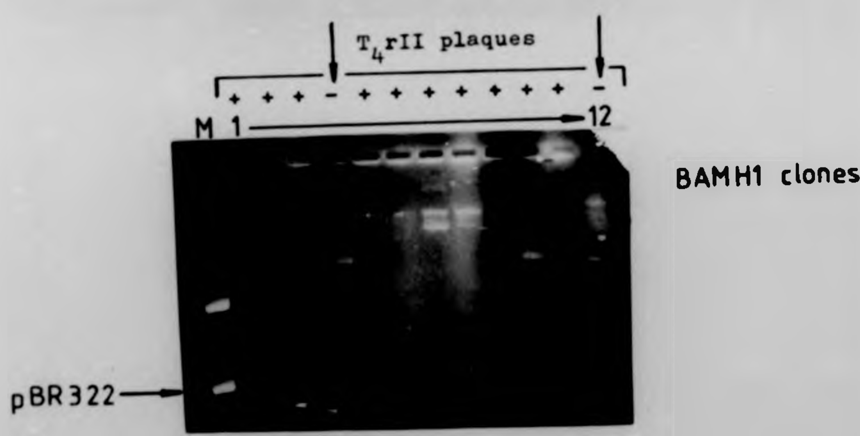
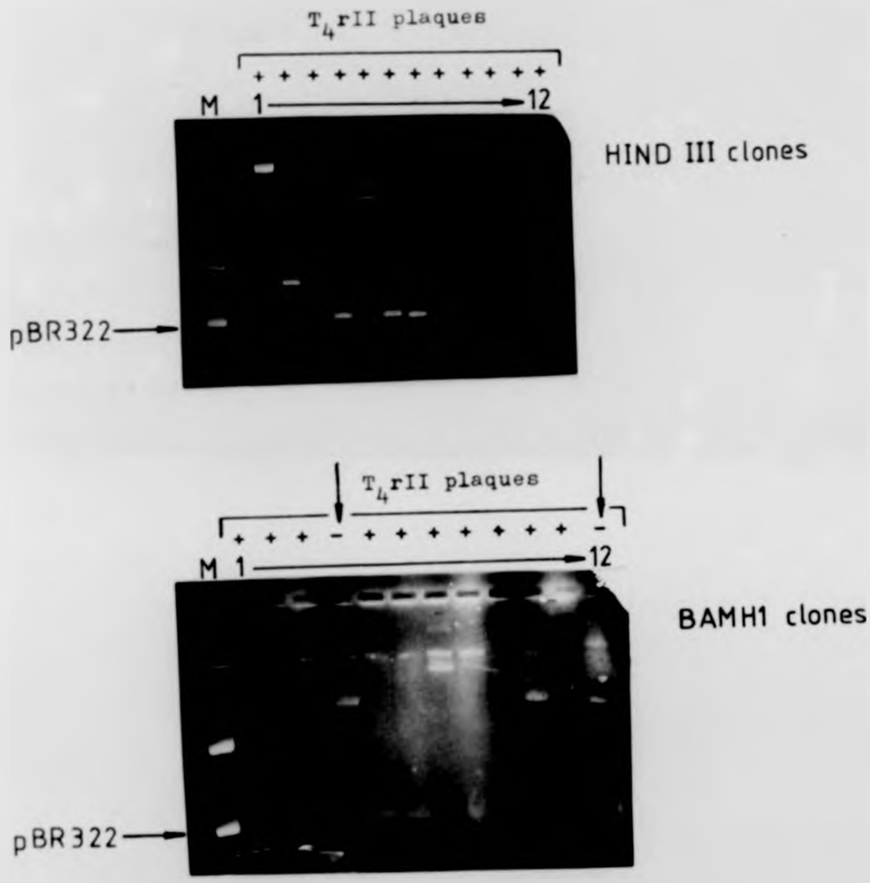

T4rII plaques
+ + + - + + + + + + + -
M 1 12
BAMH1 clones
pBR322

Figure I2. Analysis of $Ap^RTc^S$ clones produced by insertion of restriction fragments of the lambda genome into pBR322 at either the HindIII or BamHI site. In each case (BamHI and HindIII) plasmid DNA from I2 independent clones was compared to that of marker pBR322 (Track M in each case). In addition and indicated in the panel above each gel, all clones were scored for their ability to support replication of the $T_4$rII mutant H88. Two clones are indicated (pλ(Bam)4 and pλ(Bam)I2) that both contained plasmids larger than pBR322 and failed to plaque phage H88.

**Figure I2.** Analysis of $Ap^R Tc^S$ clones produced by insertion of restriction fragments of the lambda genome into pBR322 at either the HindIII or BamHI site. In each case (BamHI and HindIII) plasmid DNA from I2 independent clones was compared to that of marker pBR322 (Track M in each case). In addition and indicated in the panel above each gel, all clones were scored for their ability to support replication of the $T_4$rII mutant H88. Two clones are indicated (pλ(Bam)4 and pλ(Bam)I2) that both contained plasmids larger than pBR322 and failed to plaque phage H88.


T4rII plaques
+ + + + + + + + + + + +
M 1 12
HIND III clones
pBR322


T4rII plaques
+ + + - + + + + + + + -
M 1 12
BAMH1 clones
pBR322


T4rII plaques
+ + + + + + + + + + + +
M 1 12
HIND III clones
pBR322
T4rII plaques
+ + + – + + + + + + + –
M 1 12
BAMH1 clones
pBR322

<u>Figure 13.</u> Characterization of clone $p\lambda I2$.

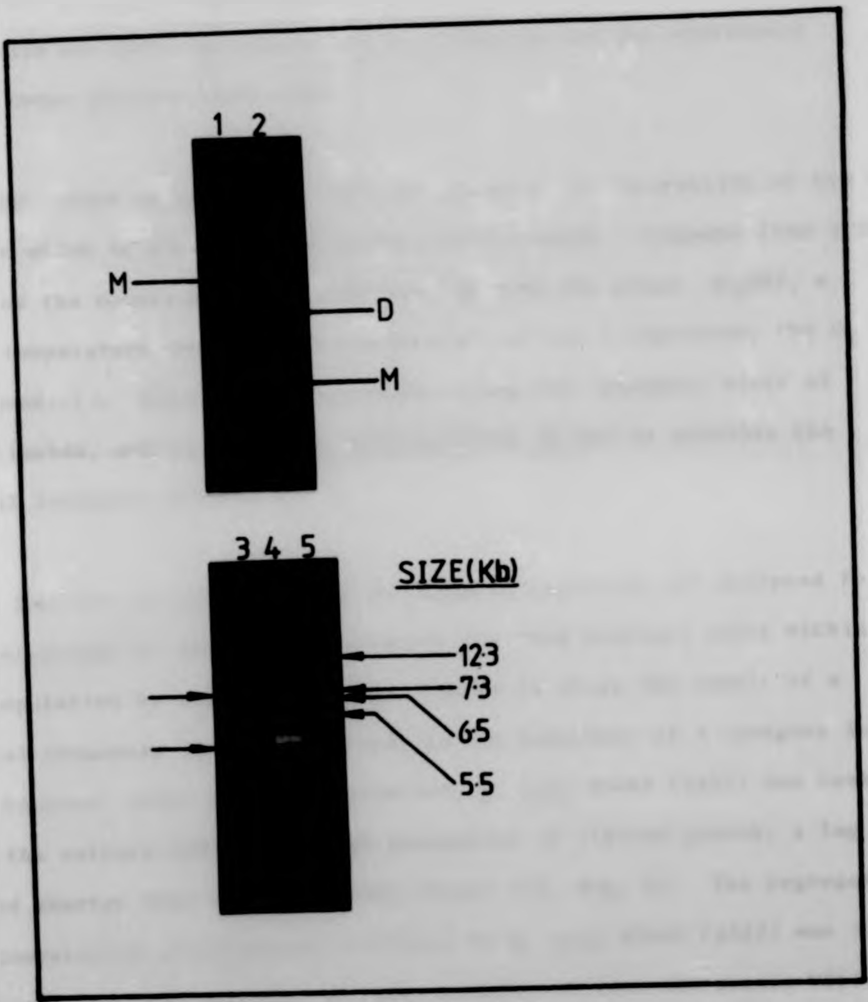
One clone (labelled $p\lambda I2$) identified as $rex^{+}$ in the tests described in the text was isolated, grown and lysed for its plasmid content. The monomeric form of $p\lambda I2$ is indicated in track 1 and compared to the monomeric and dimeric forms of pBR322 (track 2).

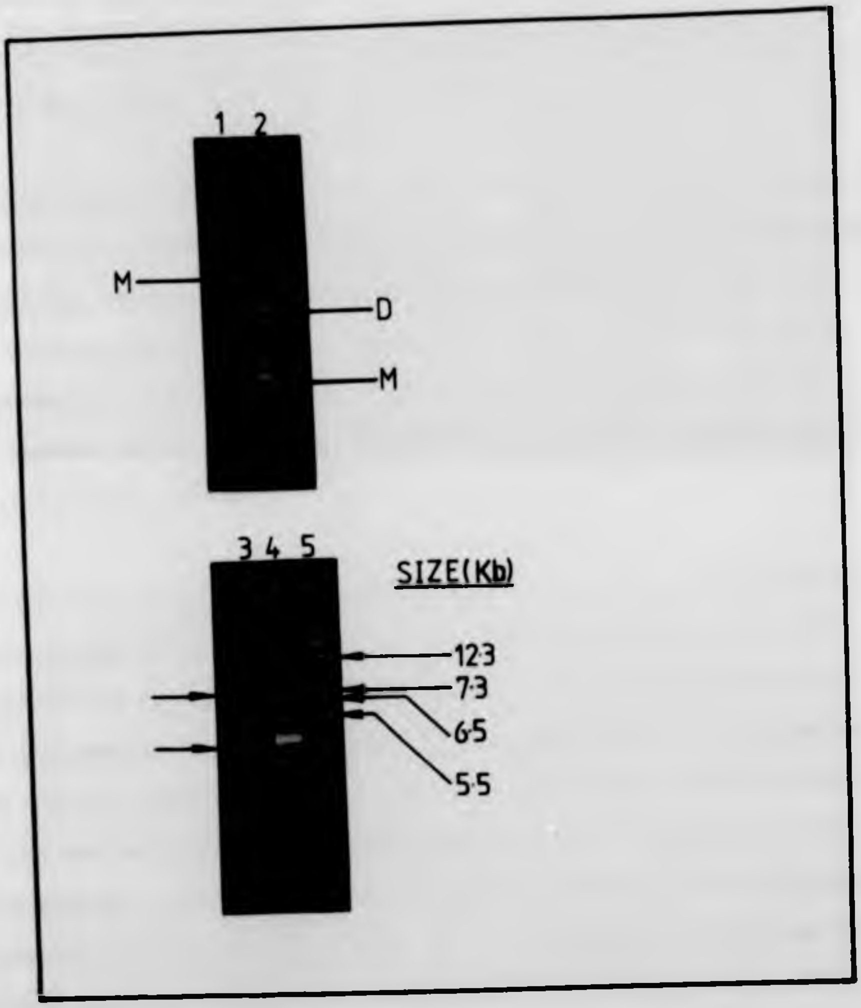
In addition, $p\lambda I2$ was incubated with the restriction enzyme BamHI and the resultant digest resolved on a 0·7 % agarose gel along with BamHI digested markers.

Track 3 - BamHI digested $p\lambda I2$ (bands are indicated).

Track 4 - " " pBR322.

Track 5 - " " lambda DNA (sizes are as shown).


1 2
M
D
M
3 4 5
SIZE(Kb)
12·3
7·3
6·5
5·5


1 2
M
D
M
3 4 5
SIZE(Kb)
12·3
7·3
6·5
5·5

iv) Chemostat cultivation of pλ12

The strain E. coli W5445 (Pλ12) was subcultured twice in the presence of ampicillin and then inoculated into a chemostat set for continuous growth under glucose limitation.

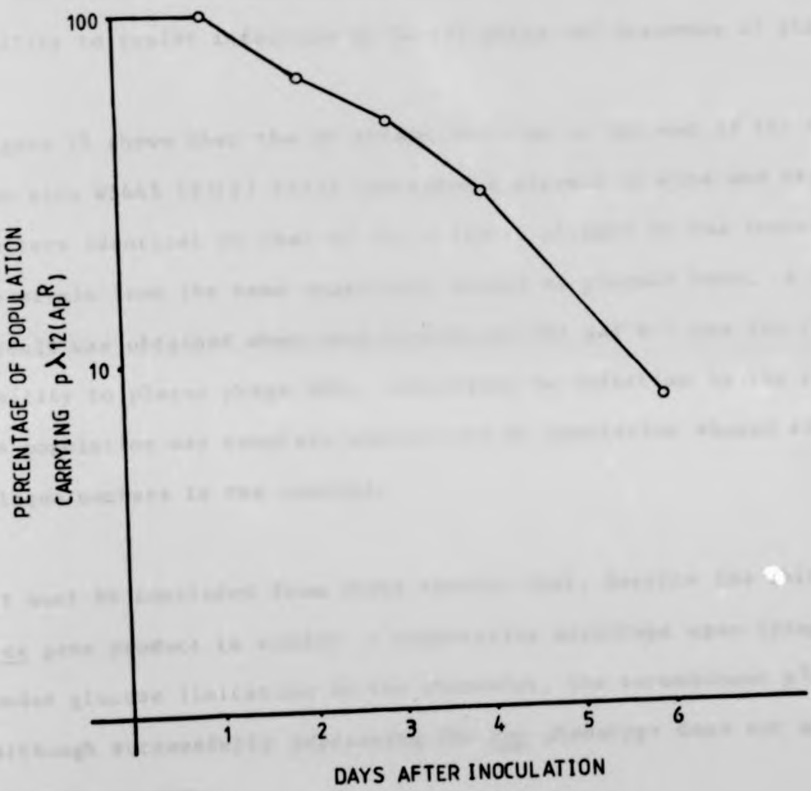
The temperature of growth was 30°C to ensure normal production of the Cl protein which is also encoded by the 7.3 Kb cloned λ fragment (the λ DNA used for the construction of pλ29 was DNA from the phage $\lambda C_I857$, a phage temperature sensitive for production of the λ repressor, the $C_I$ gene product). Repressor is produced during the lysogenic state of phage lambda, and so growth at 30°C mimicked as far as possible the natural lysogenic situation.

After inoculation the chemostat was sampled regularly and analysed for the percentage of ampicillin resistant (plasmid bearing) cells within the population by replica plating. Figure 14 shows the result of a typical chemostat run. In contrast to the behaviour of λ lysogens in the chemostat under glucose limitation, E. coli W5445 (pλ12) was lost from the culture after only 5-10 generatios of limited growth, a lag period shorter than that of pBR322 itself (cf. Fig. 6). The segregation and competitive disadvantage exhibited by E. coli W5445 (pλ12) was also exhibited when the plasmid λ12 was transferred into the strain W3110 and the resultant transformant also cultivated under glucose limitation in the chemostat (data not shown). The result indicates the strain independence of the instability exhibited by this plasmid.

Despite the earlier successful expression and transference of pλ29

Figure I4. The survival of W5445 (pλI2) in glucose limited continuous culture.

The strain W5445 (pλI2) bearing the rex gene of bacteriophage lambda was grown overnight at 30°C in IOmls of L broth supplemented with IOOμg/ml ampicillin. This culture provided the inoculum for a chemostat set for glucose limited growth at a dilution rate of $0 \cdot 2$ $hrs^{-I}$.
The culture was sampled daily and analysed for the presence of the ampicillin marker of pλI2 in the normal manner.



100
10
PERCENTAGE OF POPULATION
CARRYING pλ12(Ap^R)
1
2
3
4
5
6
DAYS AFTER INOCULATION

between strains, it remained possible that degradation of the plasmid within each host may have generated non *rex*$^+$ clones that subsequently segregated R- cells and came to dominate the culture. In order to test this the resident R+ and R- strains present in the chemostat population at the end of each experiment were isolated and examined for both their ability to resist infection by T4 rII phage and presence of plasmid DNA.

Figure 15 shows that the R+ strain isolated at the end of the chemostat run with W5445 (Pλ12) still contained a plasmid of size and restriction pattern identical to that of the original plasmid in the inoculum. The R- strain from the same experiment showed no plasmid band. A similar result was obtained when each population (R+ and R-) was sco red for the ability to plaque phage H88; resistance to infection by the remaining R+ population was complete whereas the R- population showed similar plaque numbers to the control.

It must be concluded from these results that, despite the ability of the *rex* gene product to confer a competitive advantage upon lysogen growing under glucose limitation in the chemostat, the recombinant pλ12, although successfully expressing the *rex* phenotype does not exhibit the same phenomenon.

As a consequence of the inability of pλ12 to enhance the stability of plasmid bearing cells in the chemostat, experiments with this plasmid were discontinued and a second approach to stabilization was embarked upon.

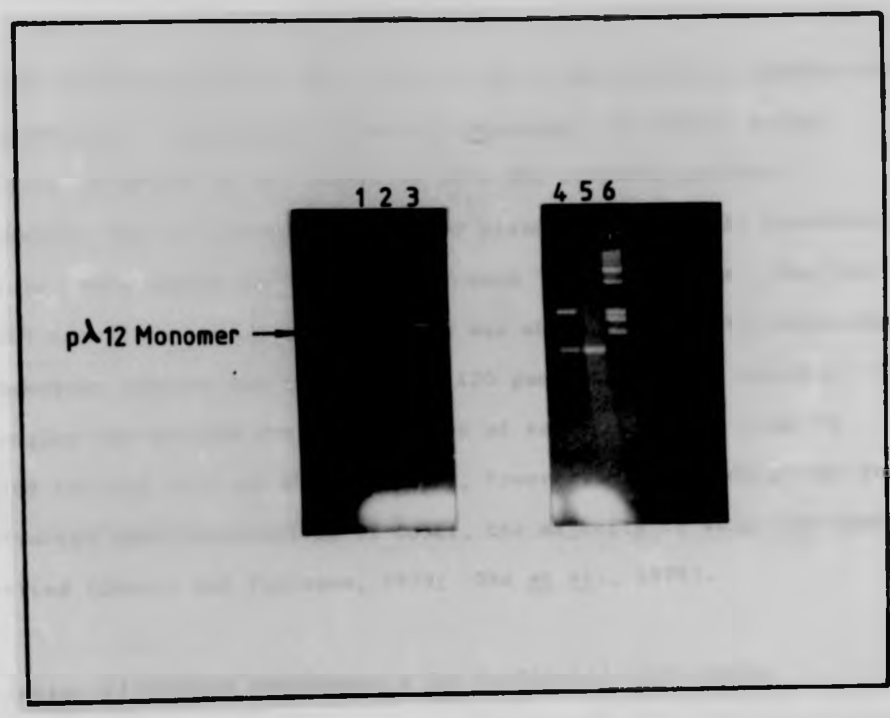
Figure I5. The plasmid content of the populations present in the chemostat after loss of plasmid p$\lambda$(Bam)I2.

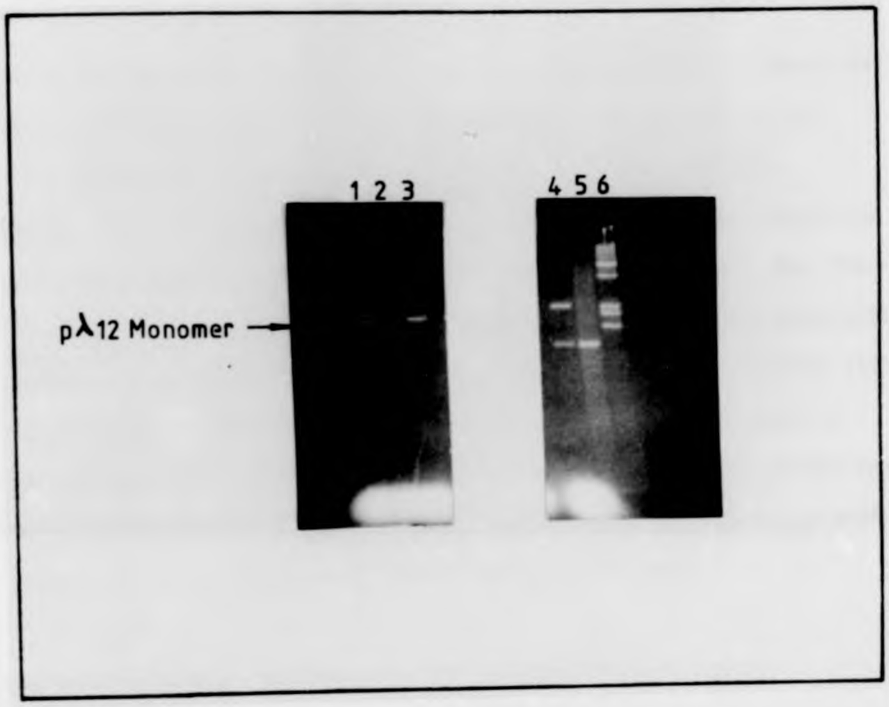
At the end of the chemostat run presented in fig.I4, representitive colonies from both the minority Ap.$^{R}$ population and the majority Ap.$^{S}$ population of the chemostat were grown in IOml cultures and lysed for their plasmid content.

Tracks I & 3 - two randomly selected Ap.$^{R}$ colonies.
Track 2 - one randomly selected Ap.$^{S}$ colony.

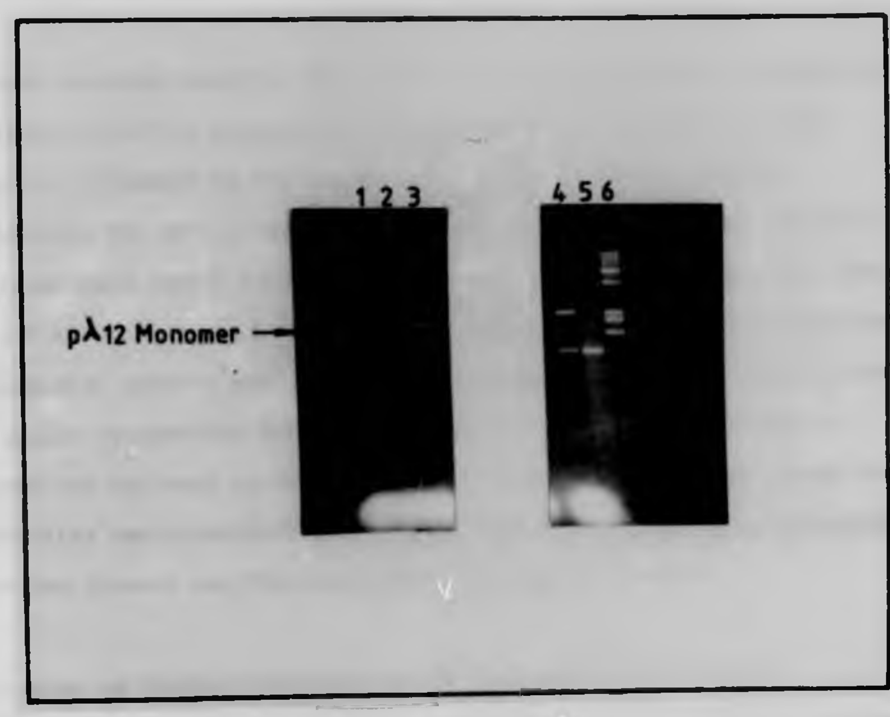
In addition, the plasmid preparation shown in track I was treated with the restriction enzyme BamHI and the resulting digest resolved on an agarose gel alongside BamHI digested markers.

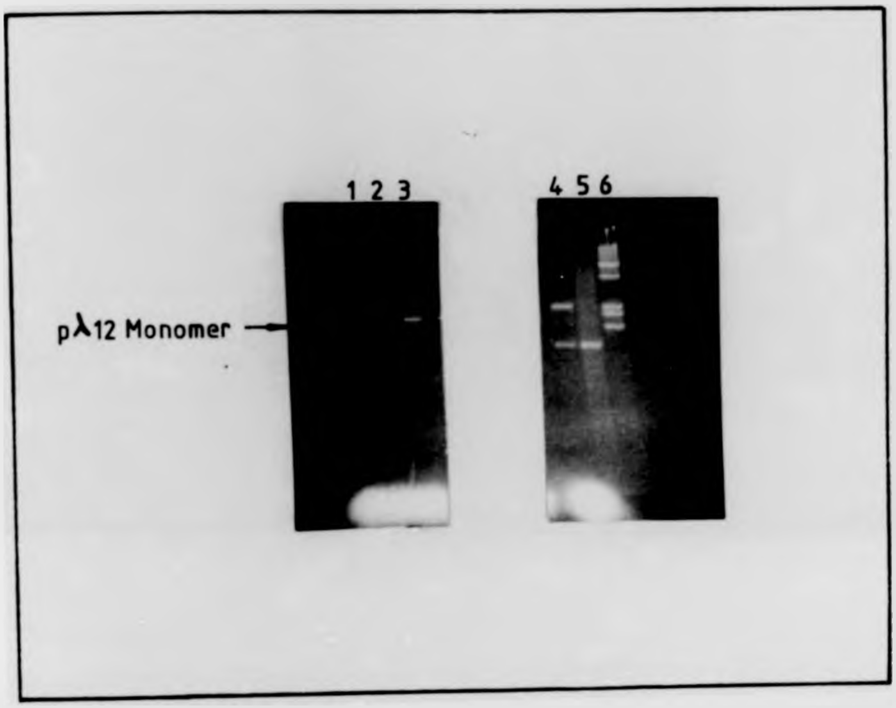
Track 4 - BamHI digested test (track I)
Track 5 - " " pBR322.
Track 6 - " " lambda DNA.


1 2 3
4 5 6
pλ12 Monomer



1 2 3
4 5 6
pλ12 Monomer


1 2 3
4 5 6
pλ12 Monomer


1 2 3
4 5 6
pλ12 Monomer

v) <u>Cloning of partition functions from pDS1109 and pPM31</u>

A second strategy used for the construction of derivaties of pBR322 with increased stability properties was to complement the defect in the stability of pBR322 by the insertion of a DNA fragment actively responsible for partitioning of another plasmid. Two stably inherited replicons were chosen as the doners of such '*par*' functions. The first, pDS1109 has already been described and was shown to be stably maintained in chemostat culture for in excess of 120 generations (cf. Results III). The region responsible for the fidelity of segregation exhibited by pDS1109 has not been accurately mapped, however, a number of groups have constructed small derivitives of ColE1, the majority of which are stably inherited (Ohmori and Tomizawa, 1979; Oka *et al*., 1979).

The region of minimum requirements for replication and stable maintenence have thus been determined and found to lie within a region of about 1 kilobase (Kb) upstream of the origin of replication. This region of DNA is convieniently contained within a single Hae II fragment of ColE1 (hence pDS1109). A detailed map of pDS1109 showing points relevant to the following construction is shown in Figure 16.

The second plasmid used for donation of partition properties to pBR322 was plasmid pPM31 and has been described by Meacock and Cohen (1980). pPM31 is a multicopy plasmid constructed to carry the *par* locus from the naturally occuring tetracycline-resistance plasmid pSC101. pPM31 was chosen for this work as, at the time of writing it was the only plasmid whose partition region (the *par* locus) had been accurately mapped and transferred between plasmids. The *par* locus was shown to lie within a

Figure I6. A physical and functional map of plasmid pDSII09.

The map was compiled from information given in:

Dougan and Sherratt, I977.

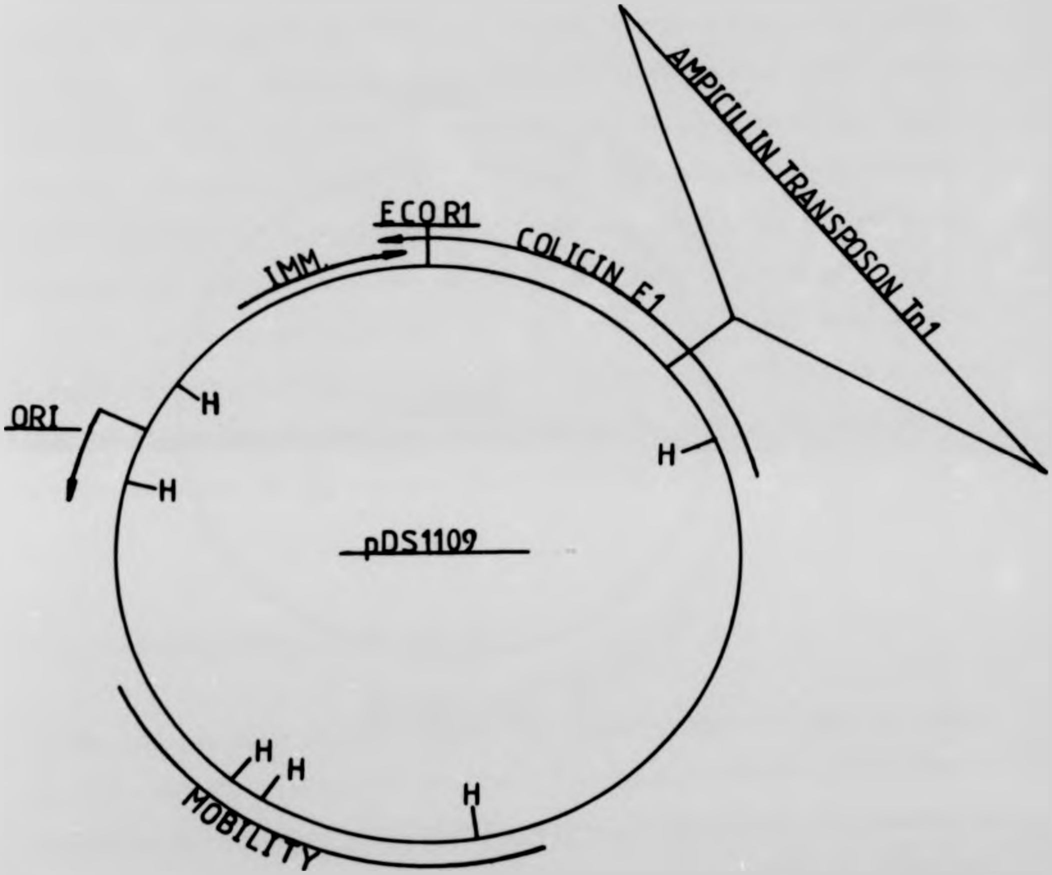
Dougan *et al.*, I978.

Ohmori and Tomizawa, I979.

Abbreviations : H - Sites for restriction enzyme Hae II.

IMM - Region coding for Colicin EI immunity protein.

ORI - Origin of replication.


AMPICILLIN TRANSPOSON Tn1
ECO R1
IMM.
COLICIN E1
ORI
H
H
H
pDS1109
H
H
H
MOBILITY

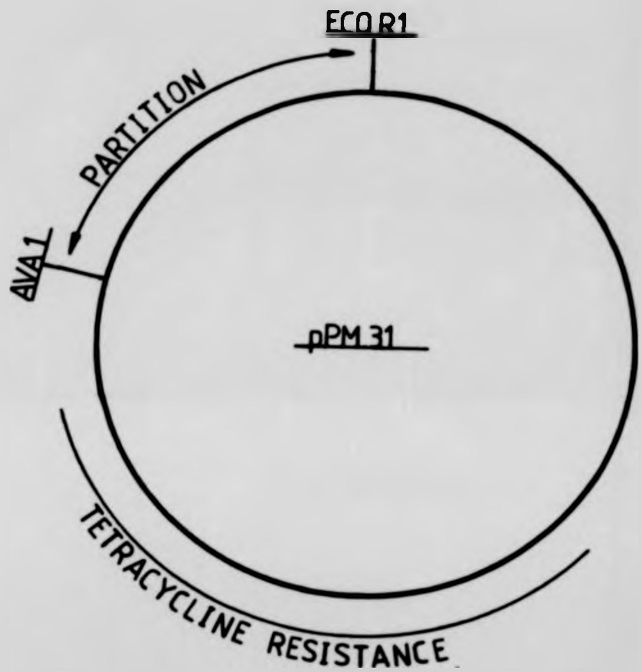



Figure 17. A physical and functional map of plasmid pPM31. The map is taken from Meacock and Cohen, 1980.

370 base pair (bp) fragment of DNA bounded by sites for the enzymes Eco RI and Ava I. The 370 bp fragment was originally found close to the origin of replication of pSC101 and lack of all or part of this region caused the segregation of pSC101-free cells within a population growing in batch culture. Since the copy number of pSC101 is relatively low (5-6 copies per genome equivalent) it could be studied without the need for continuous culture. The _par_ locus was found to act only _in cis_ and to be independent of its position on the plasmid relative to the origin of replication. In addition, and of particular interest, the _par_ locus from pSC101 was shown to confer stability on an unrelated plasmid that normally gave rise to R- cells within a population. A detailed physical and functional map of pPM31 is presented in Figure 17. The precise functional basis of _par_ action remains unknown.

vi) Cloning strategy from pDS1109

It can be seen from the map of pDS1109 shown in Figure 16 that a large (2.1 Kb) Hae II fragment of DNA that contains all necessary partition functions for ColE1 also encodes the protein responsible for immunity to Colicin E1 (see Dougan _et al_., 1978). Transformants produced after the ligation of the Hae II-A fragment of pDS1109 to pBR322 would then be resistant to the effects of Colicin E1 whilst those transformants containing only religated pBR322 would remain susceptible. This provided the basis for selection of recombinants.

The recipient plasmid pBR322 contains unique restriction sites for many enzymes but not for Hae II. A reference to the total restriction map of pBR322 (Sutcliffe, 1978) reveals the presence of 11 sites for this enzyme within the 4.3 Kb of pBR322 sequence. Fortuitously however, the majority of Hae II sites lie on the left side of the pBR322 map away from the origin of replication and the gene responsible for ampicillin resistance thus allowing the maintenance of the $Ap^r$ marker and the replication properties of pBR322 in the majority of products produced by a partial digest of pBR322 by Hae II.

The protocol followed for the construction of pBR322-Hae II-A hybrids was then to mix partially Hae II digested pBR322 with completely Hae II digested pDS1109. After ligation transformants were selected on ampicillin plates and possible recombinants found by transfer of about 1000 colonies to plates containing a lethal dose of Colicin E1.

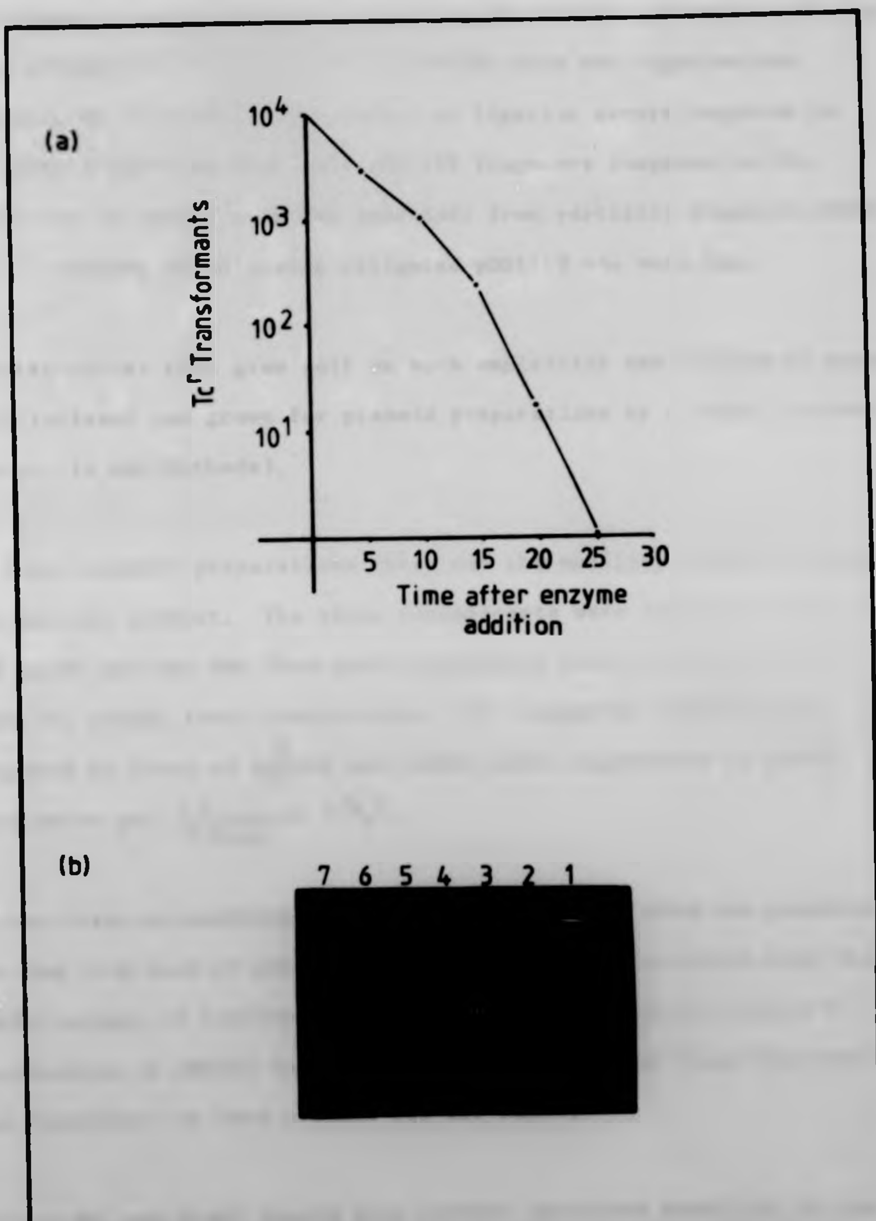
The digestion of pBR322 to give only partial products was assayed by transformation; since only circular molecules efficiently transform E. coli the generation of linear molecules by the restriction enzyme is easily monitored by this protocol. A typical result is shown in Figure 18(a). Under the conditions employed the transformation figures had dropped to zero after 30 minutes of digestion. Gel analysis (Figure 18(b)) of the DNA throughout such a digestion revealed that most of the products after 30 min of digestion were very large fragments representing pBR322 cut only once or twice. Also shown in Figure 18(b) is the complete digest of pDS1109 used for the ligation mixture. After choosing digestion times correctly the Hae II enzyme in each digestion mix was inactivated at 65°C for 10 min and the digests mixed. The concentration of ATP in the mix was adjusted to 100 μM and the fragments ligated together overnight in the presence of T4 DNA ligase.

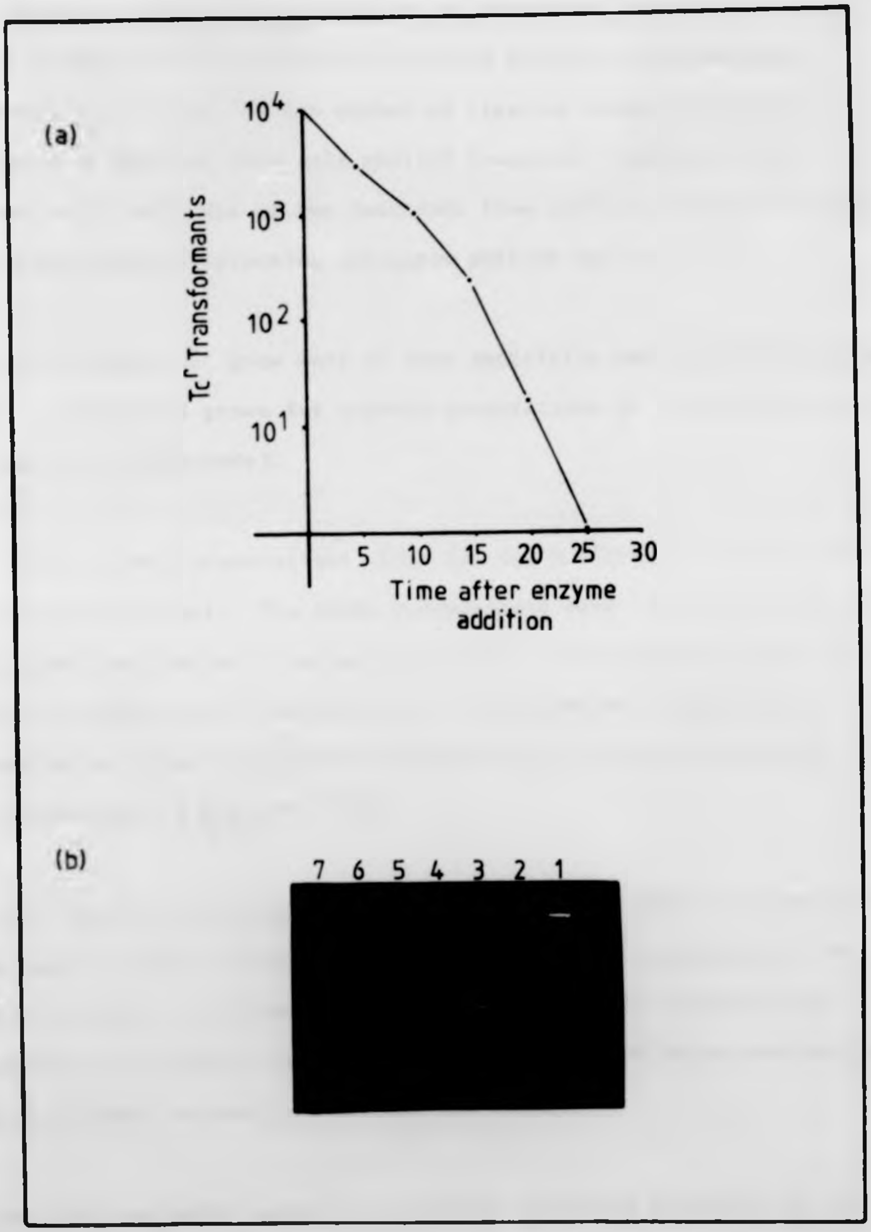
<u>Figure I8.</u> Assaying the course of a Hae II digest of pBR322. I microgram of pBR322 DNA was incubated with I unit of restriction enzyme Hae II at 37° C. Samples taken at various times after the addition of the enzyme were assayed for the presence of intact circles and physical state.

(a) Samples removed at five minute intervals after the addition of the enzyme were tested for the presence of intact circles by transformation of competent <u>E.coli</u> W5445 for tetracycline resistance.

(b) Digestion products produced by the enzyme Hae II were resolved on an 8 % acrylamide gel. Time points after addition of the enzyme were: I -0 mins, 2 - 20 mins, 3 - 40 mins, 4 - 60 mins 5 - 90 mins and 6 - I20 mins.

Track 7 shows the complete Hae II digest of pDSII09 used in the cloning experiments discussed in the text.


(a)
$10^4$
$10^3$
$10^2$
$10^1$
$Tc^r$ Transformants
5 10 15 20 25 30
Time after enzyme addition
(b)
7 6 5 4 3 2 1


(a)
$10^4$
$10^3$
$10^2$
$10^1$
$Tc^r$ Transformants
5 10 15 20 25 30
Time after enzyme addition
(b)
7 6 5 4 3 2 1

Selection of recombinants by growth on ampicillin and Colicin El would have allowed the regeneration of pDS1109 from the ligation mix. However, as a result of the number of ligation events required to generate a replicon from only pDS1109 fragments compared to the formation of only one or two junctions from partially digested pBR322, the likelihood of selecting religated pDS1109 was very low.

Several clones that grew well on both ampicillin and Colicin El plates were isolated and grown for plasmid preparations by a 'mini'-protocol (Materials and Methods).

Of those plasmid preparations three had the mobility in agarose gels of the desired product. The three recombinants were labelled pIJH0, pIJH1 and pIJH7 and the DNA from each preparation restricted with Hae II in order to reveal their constitution. The digestion products were compared to those of pDS1109 and pBR322 after separation on an 8% acrylamide gel (figure 19).

Of the three plasmids digested, two (pIJH1 and H7) show the presence of the Hae II-A band of pDS1109. Only pIJH7 however retained only the pBR322 origin of replication. pIJH1 contained both the origin of replication of pBR322 and that of pDS1109, which of these two origins was functional on this plasmid was not known.

Both pIJH1 and pIJH7 should thus exhibit increased stability in the chemostat if a '*par*' function is present on the Hae II-A fragment of pDS1109. To test this both plasmids were transferred afresh into *E.*

Figure I9. Characterisation of Colicin EI immune clones. Plasmid DNA was extracted from each of three clones described in the text and digested by the enzyme Hae II. Digestion products were seperated on an 8 % polyacrylamide gel and compared to Hae II digested pBR322 and pDSII09.

Marker sizes are those published for pBR322 and bands of interest discussed in the text are indicated.

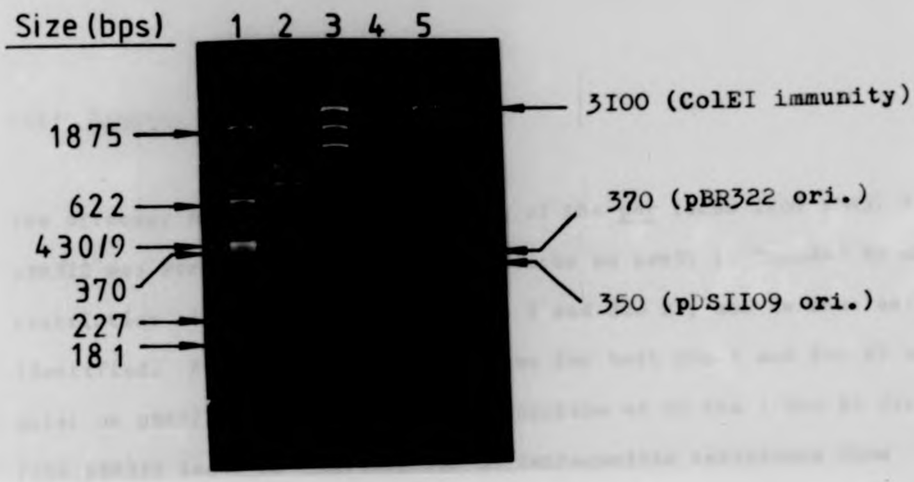
Tracks are : I - pBR322 digested by Hae II.

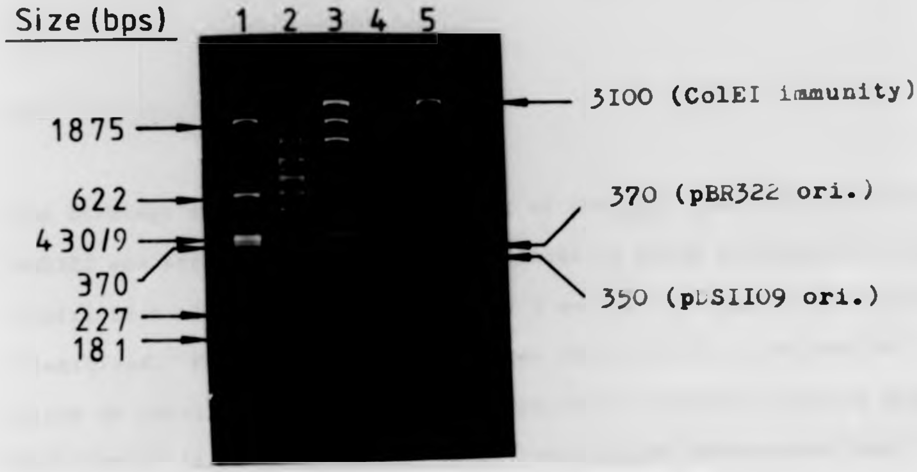
2 - pDSII09 " " "

3 - pIJH O " " "

4 - pIJH 7 " " "

5 - pIJH I " " "


Size (bps)
1 2 3 4 5
1875
622
430/9
370
227
181
3100 (ColEI immunity)
370 (pBR322 ori.)
350 (pDSI109 ori.)


Size (bps)
1 2 3 4 5
1875
622
430/9
370
227
181
3100 (ColEI immunity)
370 (pBR322 ori.)
350 (pLSII09 ori.)

coli W5445 and their segregational stabilities tested by continuous cultivation

vii) Cloning strategy from pPM31

The strategy employed for the cloning of the par locus from pPM31 into pBR322 was straightforward. The par locus on pPM31 is bounded by unique restriction sites for the enzymes Ava I and Eco RI, and is thus easily identified. Fortuitously, unique sites for both Ava I and Eco RI also exist on pBR322, and, in addition, insertion of an Ava I/Eco RI fragment into pBR322 leads to inactivation of tetracycline resistance thus allowing easy selection of recombinants.

Both plasmids pPM31 and pBR322 were restricted to completion by enzymes Ava I and Eco RI. Enzymes were inactivated by heat and the digests mixed and adjusted to 100 µM ATP. Ligation was started by the addition of T4 DNA ligase and continued overnight.

Transformants from such a ligation mixture were selected on ampicillin-plates and replica-plated onto plates containing tetracycline. The larger fragment of the two produced by Ava I - Eco RI digestion of pPM31 also encodes tetracycline resistance (see Figure 17) thus, selection of tetracycline sensitive clones not only selects against regligation of pBR322, but also against incorporation of the large Ava I-Eco RI fragment of pPM31. Since Ava I and Eco RI ends cannot rejoin without an insert, tetracycline sensitive clones can only be the desired product.

Several clones that were $Ap^r$ $Tc^s$ were selected and their plasmid content examined by a 'mini' extraction procedure. The majority of clones contained a plasmid slightly smaller than pBR322 as expected; the Eco RI-Ava I fragment lost from pBR322 was 1.4 Kb whilst that added was only 370 bps, a net loss of some 1.1 Kb.

One clone, pIJ005 was further analysed by restriction enzymes. Figure 20 shows the Ava I and Eco RI digestion products of pBR322, pPM31 and pIJ005. The par containing small Eco-Ava fragment from pPM31 is present in pIJ005 whilst the larger band comigrates with the large Ava I-Eco RI fragment of pBR322. pIJ005 is thus the desired ligation product and a sample of this DNA was transformed into E. coli W5445 and maintained as a stock culture prior to growth in the chemostat.

viii) The hereditary stability of plasmids pIJH7, pIJH1 and pIJ005

a) pIJH7

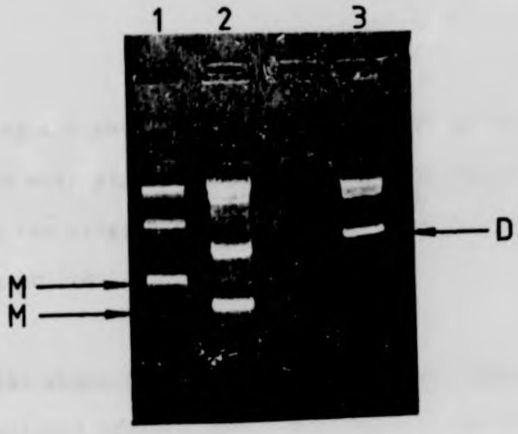
E. coli W5445 (pIJH7) was grown to expontial phase in L-Broth prior to the inoculation of a glucose-limited chemostat operated in the normal way. Results presented in Results III showed that a culture run of about 80 generations was sufficient to allow the detection of R-segregants of pBR322 derived plasmids. The culture of W5445 (pIJH7) was thus set at a dilution rate of 0.25 $hr^{-1}$ and sampled regularly through the following two weeks (about 80 generations). Samples were analysed for plasmid content in the normal way. Under similar conditions of growth W5445 (pBR322) had segregated R- cells within 40 generations but, as shown in Figure 21(a) no segregants of pIJH7 were

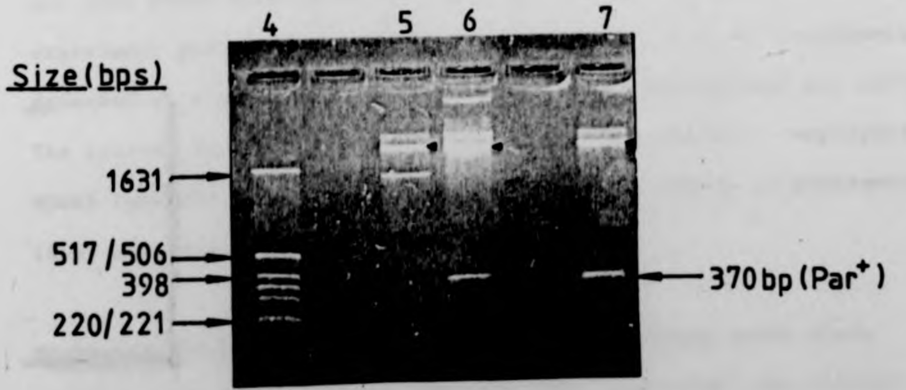
Figure 20. Characterisation of plasmid pIJ005.

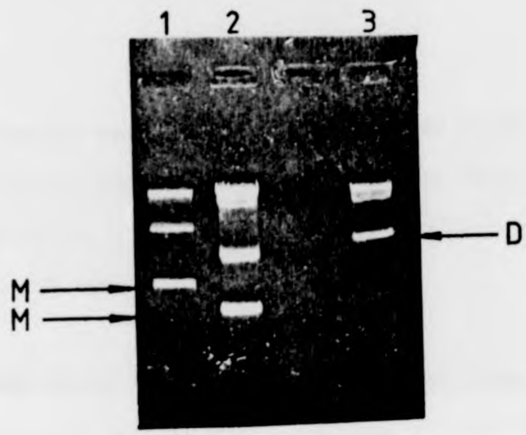
Plasmid DNA from one $Ap^R Tc^S$ clone (pIJ005), produced as described in the text, is shown in comparison to both pBR322 and pPM3I. Track-I; pBR322, Track-2; pPM3I, Track-3; pIJ005. M. and D. indicate the position of monomeric or dimeric forms of each plasmid. In addition, each plasmid is shown digested with the enzymes AvaI and EcoRI. In each case the digest is not quite complete and the final large AvaI - EcoRI fragment is indicated (◄).

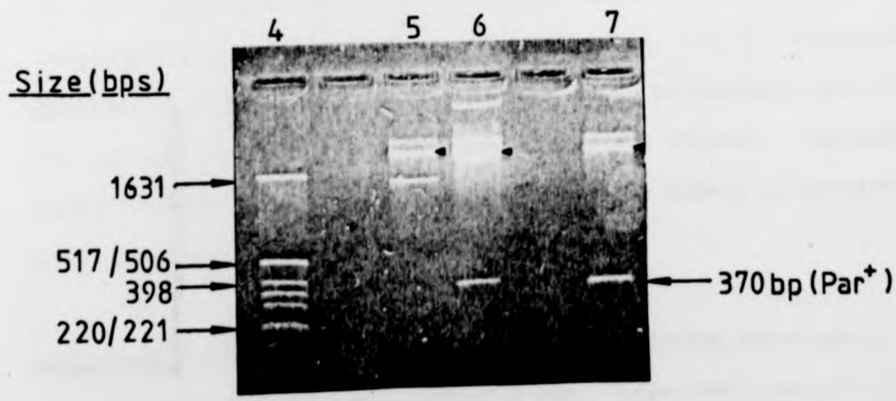
Track-5; Hinf I digested pBR322 to provide markers of sizes shown. Track-6; pBR322 digested by AvaI & EcoRI. Track-7 pPM3I digested by AvaI & EcoRI. Track-8; pIJ005 digested by AvaI & EcoRI.

Uncleaved and cleaved DNAs' were separated on agarose gels of 0·5% and I·2% respectively.


1
2
3
M
M
D


4
5
6
7
Size(bps)
1631
517/506
398
220/221
370bp (Par+)


1
2
3
M
M
D


4
5
6
7
Size(bps)
1631
517/506
398
220/221
370bp (Par⁺)

detected throughout the 80 generations of growth.

This experiment was repeated several times with the same outcome.

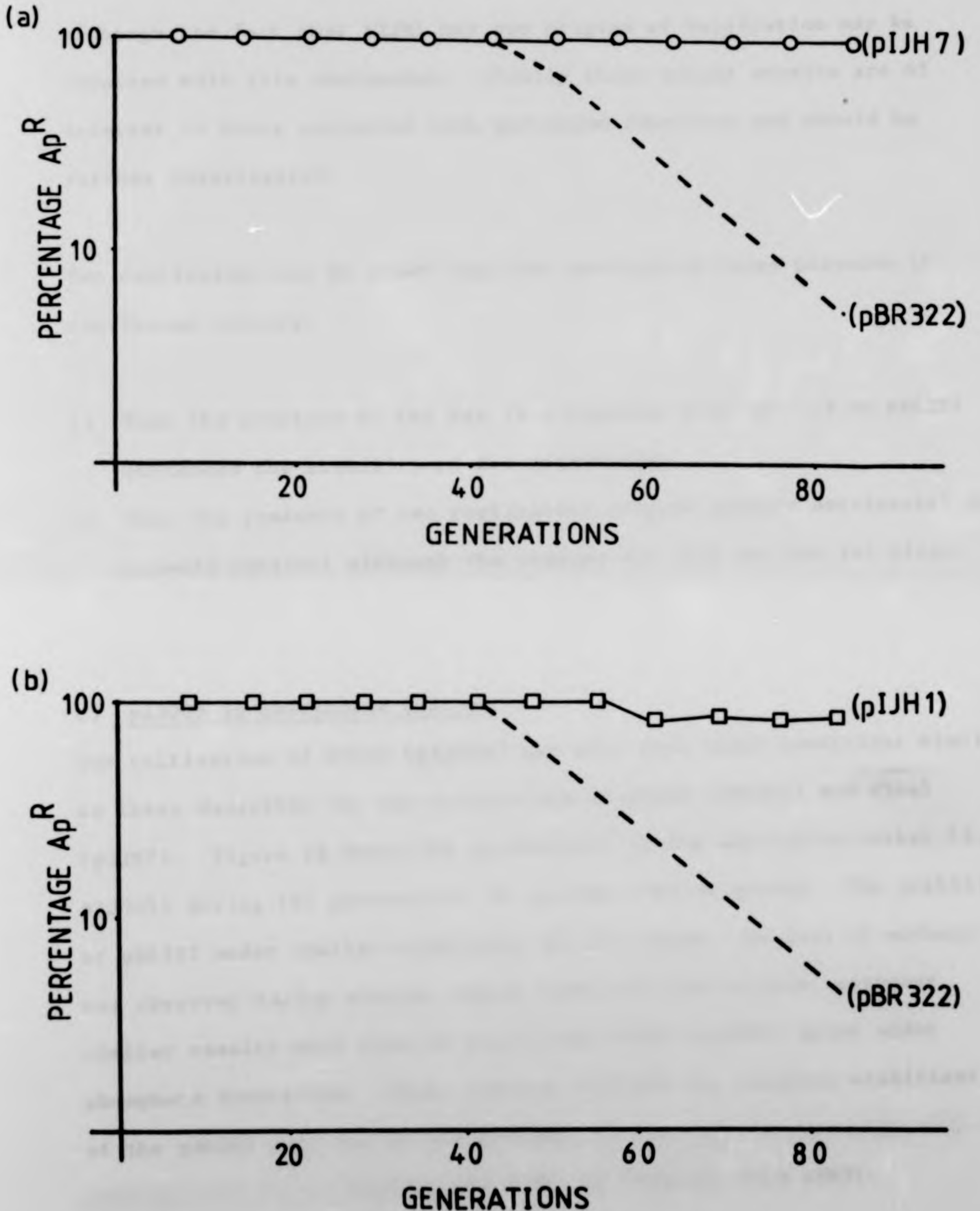
b) pIJH1

After noting the above results with plasmid pIJH7, a similar pattern was anticipated with pIJH1, a similar construct (Hae II A positive) but containing two origins of replication, one derived from pBR322, the other from pDS1109.

Figure 21(b) shows the results of a similar long period of cultivation (80 generations) using pIJH1. Contrary to expectations, R- segregants arose late on in the life of the culture (when the copy number was low) but surprisingly failed to 'takeover' the culture; their numbers did not rise above 0.5%-2% of the total population. Repetition of this experiment gave similar patterns of segregation, i.e. R- segregants were detected at a basal level and did not come to predominate the culture. The reasons for the failure of competition are obscure; segregants of W5445 (pBR322) came to predominate the culture within 10 generations of their reaching a detectable number.

Chemostat theory predicts that, if a mutant arising under these conditions is truly isogenic then it must 'take-over' the culture (Powell, 1958). Thus it must be concluded that the R- segregants arising from strains of W5445 (pIJH1) are not truly isogenic. The reason why such mutants should have arisen under cultivation of W5445 (pIJH1) and not with the other stable plasmids examined is not known

<u>Figure 2I.</u> The survival of <u>E.coli</u> W5445-(pIJHI) or -(pIJH7) in glucose limited continuous culture. In each case the presence of the ampicillin resistance marker of pIJH7 (fig.2I a) or pIJHI (fig.2I b) is shown in comparison with results typical of pBR322 (cf. fig.6). In both experiments the dilution rate was $0{\cdot}2hrs^{-I}$ (a mean generation time of about 3 hours).


(a)
100
10
PERCENTAGE $Ap^R$
20
40
60
80
GENERATIONS
(pIJH7)
(pBR322)
(b)
100
10
PERCENTAGE $Ap^R$
20
40
60
80
GENERATIONS
(pIJH1)
(pBR322)

although the fact that pIJH1 has two origins of replication may be involved with this phenomenon. Clearly these mutant strains are of interest to those concerned with partition functions and should be further investigated.

Two conclusions can be drawn from the survival of these plasmids in continuous culture:

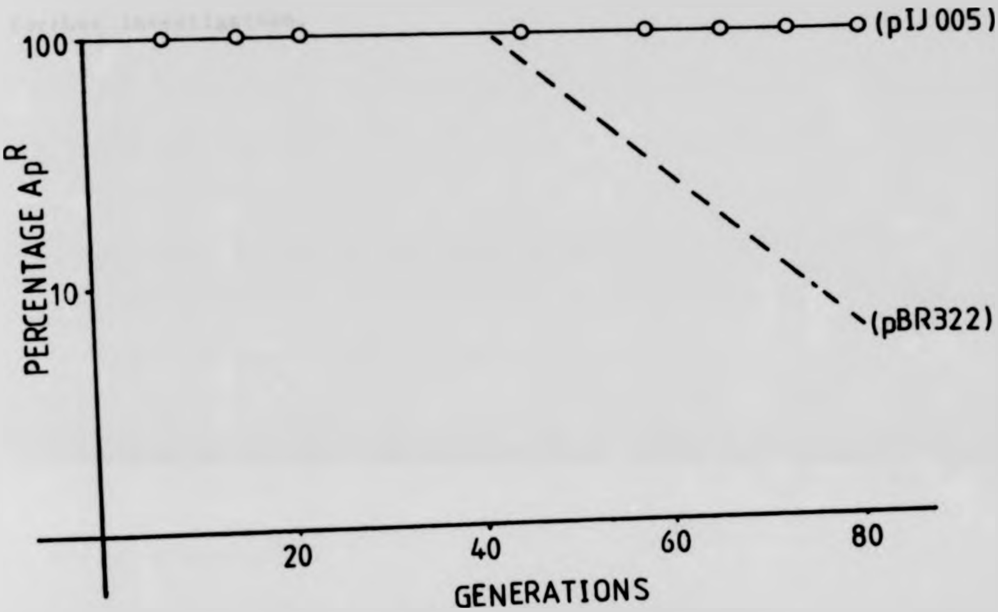
1) That the presence of the Hae II A fragment from pDS1109 on pBR322 enchances the stability of the recombinant.
2) That the presence of two replicative origins appears detrimental to plasmid survival although the reasons for this are not yet clear.

c) pIJ005 in continuous culture

The cultivation of W5445 (pIJ005) was also done under conditions similar to those described for the cultivation of W5445 (pBR322) and W5445 (pIJH7). Figure 21 shows the persistence of the ampicillin marker (i.e. pIJ005) during 120 generations of glucose limited growth. The stability of pBR322 under similar conditions is also shown. No loss of markers was observed during several repeat runs with this strain, moreover, similar results were also obtained with W5445 (pIJ005) grown under phosphate limitation. These results indicate the complete stabilization of the pBR322 replicon by the presence of the *par* bearing Ava I-Eco RI fragment from pPM31.

In agreement with the results of Meacock and Cohen (1980), it is clear that the *par* locus from pSC101 maintains its ability to ensure efficient

Figure 22. The survival of E.coli W5445(pIJ005) in glucose limited continuous culture. After inoculation the chemostat was set to a dilution rate of 0·24 $hrs^{-1}$ (a mean generation time of ~3 hours) and sampled daily. The presence of the ampicillin marker (O) throughout the course of the run is shown compared to results typical for pBR322 (cf. figure 6).


(pIJ 005)
100
10
PERCENTAGE ApR
(pBR322)
20
40
60
80
GENERATIONS

segregation of daughter molecules, even when cloned into unrelated replicons. Exactly how such a small DNA fragment is able to dramatically alter the segregational properties of pBR322 is worthy of further investigation.

# V Discussion of Results presented above : Stability of plasmids in Continuous Culture

i) Stability of RP4

ii) Stability of pDS1109 and pBR322 in continuous culture

iii) Stabilization of pBR322 in continuous culture

iv) Nature of the 'par' function and overall conclusions

v) Final conclusions

i) Stability of RP4

The experiments done in this thesis started as an extension of the work of Melling et al. (1977). Their work, based on the plasmid RP1 was repeated with the related plasmid RP4 and produced similar results. In addition, the period of growth in the absence of selective pressure was increased under the most stringent nutrient-limited conditions; no loss of markers was observed in up to 200 generations of continuous growth. In reconstitution experiments, the possibility of self selection of plasmid carrying cells was ruled out by the demonstration, under phosphate-limited conditions of competition between R+ and R- populations.

These results imply two conclusions; firstly that no fragmentation of the plasmid or at least of those markers tested, occurred during long periods of growth in the absence of selection. This result is in agreement with that published by Wouters et al. (1978) for the stability of plasmid R6 in continuous culture but differs from that reported by Godwin and Slater (1979) for the stability of Tp120 in continuous culture. Individual marker loss is thus probably a property of the plasmid structural stability within an individual cell. For example, N group plasmids of which Tp120 is one, are known to dissociate markers in complex media under batch culture (Pinney et al., 1977).

Secondly, even under conditions known to select for plasmid free cells, none were detected, implying the existence of a mechanism that ensures segregation of plasmid molecules into daughter cells at division.

What could be the basis of such a mechanism?

i) Stability of RP4

The experiments done in this thesis started as an extension of the work of Melling *et al*. (1977). Their work, based on the plasmid RP1 was repeated with the related plasmid RP4 and produced similar results. In addition, the period of growth in the absence of selective pressure was increased under the most stringent nutrient-limited conditions; no loss of markers was observed in up to 200 generations of continuous growth. In reconstitution experiments, the possibility of self selection of plasmid carrying cells was ruled out by the demonstration, under phosphate-limited conditions of competition between R+ and R- populations.

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Secondly, even under conditions known to select for plasmid free cells, none were detected, implying the existence of a mechanism that ensures segregation of plasmid molecules into daughter cells at division.

What could be the basis of such a mechanism?

Large plasmids such as RP4 or RP1 are commonly maintained at low copy number within E. coli and it is thus unlikely that equipartition of daughter molecules is effected as a consequence of the random distribution of plasmids at division. Rather, as has been demonstrated or implied for other large low copy number plasmids (P1, Sternberg and Austin, 1981; R1, Nordstrom et al., 1980; R6-5, Timmis et al., 1981) it is reasonable to assume a specific locus, 'par' (for partition) or 'stb' (for stability) as being involved in the active partitioning of plasmids into newly dividing cells.

The need for an efficient partitioning system is obvious when plasmid copy numbers are low; maintenence of a plasmid cannot be guaranteed by random plasmid assortment, but this is not necessarily the case for plasmids whose copy number is high. Indeed, it has been argued (Broda, 1980) that high copy number is a mechanism of efficient plasmid segregation.

How then would high copy number plasmids behave in long periods of continuous culture?

ii) Stability of pDS1109 and pBR322 in continuous culture

Conditions established for the demonstration of competition were shown to allow R- population detection from low levels in a variety of E. coli cultures harbouring plasmid pBR322 and were thus used to test the long term stability of pBR322 and another related multicopy plasmid pDS1109. Despite 80 generations of stringent nutrient-limited growth, no R-segregants were detected in cultures of E. coli harbouring pDS1109, whereas segregants of *E. coli* (pBR322) were detected within 40 generations of continuous growth. pBR322-free cells were shown to appear after about 30-40 generations of growth, irrespective of host genetic background, and subsequently shown to rise to 'takeover' the culture with slower than normal kinetics. This observation was intepreted as a decline in the copy number of pBR322 prior to the segregation of R- cells.

The effect of long term culture on copy number was then examined directly for pDS1109 throughout a period of 82 generations of continuous culture. Irrespective of the method employed to measure copy number, its value was shown to decrease by a factor of between 4 and 5 fold throughout the growth period tested. The reduction in copy number observed for pDS1109 and implied for pBR322 suggests that plasmid replication is limited to some extent by the availability of carbon and energy. In cellular terms, this can be considered 'good housekeeping' but it implies that although the maximum number of plasmid copies per cell is determined genotypically (Twigg and Sheratt, 1980; Shepard *et al.*, 1979) the actual copy number within any one organism may be dependent on phenotypic pressure from the environment. In the natural environment for example, growth is more often than not limited by available nutrients and it is thus unlikely that many multicopy plasmids reach their maximum copy numbers. It follows then that segregation of plasmids into daughter cells cannot always be guaranteed simply as a consequence of high copy number. One might expect therefore that the active partition elements suggested for RP4 and RP1 are also present on multicopy plasmids such as pDS1109. This is indeed borne out by experimentation for despite the low copy number of pDS1109 towards the end of the chemostat run no R- segregants were ever detected.

The observation that pDS1109 was stably maintained in chemostat culture whereas pBR322 and its progenitor pMB9 were not, implies that pBR322 has lost a function normally encoded by ColE1 type plasmids (witness the stability of pDS1109) that is responsible for the fidelity of segregation.

Plasmids pBR322 and pDS1109 share many biological properties, both exist at a relatively high (though controlled) copy number (Twigg and Sheratt, 1980; Shepard _et al._, 1979) and as far as has been determined, their requirements for replication are the same (Bolivar _et al._, 1977). Furthermore, both molecules replicate unidirectionally from a well defined origin, which has in each case been sequenced and shown to be essentially the same (Bastia, 1977; Sutcliffe, 1979). It is reasonable to propose therefore, that any differences between pDS1109 and pBR322 that result in the observed changes in hereditary stability lie outside of the origin area.

Downstream (i.e. in the direction of movement of the replication fork) from the replicative origin lie the sequences responsible for binding the proteins involved in the relaxation complex. The fact that ColK (pDS4101) does not increase the hereditary stability of pBR322 when supplied _in trans_ suggests that the relaxation complex or at least those parts of it complementable by ColK, is not involved in segregation fidelity.

Upstream of the replicative origin of pBR322 the polynucleotide sequence differs to some small extent from that determined for ColEl (Sutcliffe, 1979). Moreover, this region has been suggested as a region functional in the separation of daughter molecules after a round of replication (Sakakibara, 1976). Any partition element present may be expected to act similarly at this time (i.e. at the end of a replication cycle) and it is possible that these two functions map closely together or overlap. In this regard it is noteworthy that sequence differences observed between pBR322 and ColEl have been shown to alter possible open reading frames (i.e. possible protein products) in the exact area suggested as active in molecule separation by Sakakibara (1976) (see Ohmori and Tomizawa, 1979). Under conditions of high copy number the activity of any 'par' function would be largely redundant; random assortment would lead to efficient segregation. However, under conditions of low copy number, as is the case after many generations in nutrient limited culture the activity of such an element would be essential for efficient plasmid segregation. This evidently occurs in the case of pDS1109 but not pBR322.

An alternate variation to this theme assumes that the base changes observed between pDS1109 and pBR322 are inconsequential and are not the cause of the _par_ phenotype exhibited by pBR322. Rather, the region involved in _par_ action is completely absent from pBR322 as a consequence of the drastic size reductions employed during its construction (Sutcliffe, 1979). In either case there is some corroborative evidence to suggest that the _par_ function lies upstream of the origin of replication.

Many groups have been interested in the replication of ColEl and, in the course of these studies a number of mutant or deleted plasmids have been constructed with a view to defining the minimum region required for replication. Inheritability of such mutant plasmids in the absence of selective pressure is rarely discussed, but occasionally plasmids are noted as particularly unstable. Oka *et al*. (1979) deduced the nucleotide sequence for a large region of ColEl upstream of the origin of replication with the aid of mini-ColEl derivitives. One such derivitive (pA07) was noted as unstable in the absence of selective pressure although when selection was applied the plasmid replicated normally, suggesting the lesion did not affect DNA replication. When pA07 was mapped it was found to have lost sequences beyond a point about 400 base pairs upstream of the replicative origin.

Heffron *et al*. (1978) reported the isolation of a mutant ColEl by the integration at random positions of a synthetic oligonucleotide encoding the restriction site for the enzyme Eco RI. The position of the integrations was simply determined by digestion of the plasmid by Eco RI and another 'landmark' enzyme. One mutant so generated exhibited instability under normal growth conditions and the mutation was mapped to the region of ColEl upstream of the origin of replication. Finally, Inselberg (1981) isolated a series of mutants by hydroxylamine mutagenesis that exhibited instability. Three such mutants were mapped upstream of the replicative origin.

In all these cases, when selection was applied replication was observed as normal indicating that plasmid replication *per se* was not the site of the mutagenic event. It is possible that some of these mutants reduced copy numbers to such an extent that segregation was a frequent event, but since segregation is not the necessary consequence of low copy number (witness the results in continuous culture with pDS1109) then partition functions may have also been affected.

All the preceeding examples demonstrate the probable position of the ColE1 'par' element postulated in the work presented in this thesis as lying in the region of DNA upstream of the origin of replication and maintained in the largest fragment of ColE1 DNA bounded by sites for the restriction enzyme Hae II (i.e. the Hae II A fragment) and it was logical therefore to attempt the stabilization of pBR322 inheritance by suppying *in cis* the ColE1 Hae II A fragment. In addition, other attempts of stabilization were made.

iii) Stabilization of pBR322 in continuous culture

a) Two approaches were attempted to correct the apparent defect in partitioning exhibited by pBR322. The first is more correctly thought of as an attempt to continually select for plasmid carrying cells rather than a correction of the partitioning function *per se*. As described in an earlier section the *rex* gene product has been shown to give a competitive advantage to lysogens as opposed to non-lysogens when grown in glucose limited chemostat culture. The *rex* gene was therefore cloned onto pBR322 using the restriction enzyme Bam HI. The recombinant plasmid so derived (p$\lambda$12) was then grown in continuous culture under glucose limitation. This approach to stabilization was concluded unsuccessful when p$\lambda$12 was observed to be lost from glucose limited chemostats with faster kinetics than pBR322 itself. Moreover, p$\lambda$12 was lost after only about 5 generations of growth, a result that probably reflects the lower copy number of p$\lambda$12 compared to pBR322 as a consequence of its increased molecular weight.

The reasons for the inability of the cloned rex gene to confer upon its host the same advantage as that exhibited by $\lambda$ lysogens are unclear, but two immediate differences between the two systems may, either singularly or together, account for the failure of p$\lambda$12 to exhibit increased competitive fitness in the chemostat.

1) As noted in an earlier section, the lysogenic state requires little in the way of additional cellular machinery for its maintenence. Apart from the additional replication requirements of the $\lambda$ genome only two genes are active in the lysogenic state (repressor and rex). This situation contrasts sharply with the cell bearing p$\lambda$12 where the ampicillin, CI and rex gene products are being made and are present at several copies per cell. Clearly the maintenence requirement of this latter strain is greater than that of the lysogen and may outweigh any advantage gained by the increased efficiency of glucose metabolism permitted by rex.

2) Although rex mutant lysogens do not exhibit the same competitive advantage as wild type $\lambda$ lysogens in the chemostat (thus implicating the rex gene product in the effect) there is no evidence to suggest that the rex gene product alone confers this advantage. It is possible that the isolation of the rex gene by the cloning procedure described here has denied the gene product of an involvement or

interaction with other lambda functions necessary for the observable increase in fitness.

The lack of success with pλ12 prompted a series of cloning experiments to correct the *par* function of pBR322 at source, that is to say to provide known 'par' functions *in cis* to ensure efficient segregation.

b) Plasmids pIJH1, H7 and 005

Both plasmids pIJH1 and pIJH7 contained the pBR322 origin of replication and, in addition the Hae II-A fragment of pDS1109. This fragment has been suggested as the DNA fragment bearing the 'par' function of ColE1 (see Chapter V(ii) and VI(iii)). pIJH1 also contained the fragment of pDS1109 bearing the ColE1 origin of replication. As a result of the cloning protocol (from pBR322 partial digests) the origin of pBR322 is likely to have been retained intact with respect to its normal position on the plasmid, it was thus considered to be functionally active. Whether or not the included origin fragment of ColE1 was active was not determined. Both pIJH1 and pIJH7 were grown in continuous culture under conditions known (cf. Results II and III) to detect R- segregants of pBR322 within about 40 generations of growth. Neither plasmid showed R- segregants within the first 80 generations of growth, suggesting at least some degree of stabilization. However, on prolonged culture (up to 120 generations) regardless of the nutrient limiting regime, W5445 (pIJH1) gave rise to plasmid-free cells. Such segregants were judged, by virtue of their non-competitive behaviour to be not truely isogenic although their exact nature is not known. The reasons why such mutants should have arisen with this plasmid and not with any other must await further characterisation of the mutants themselves and the exact structure of pIJH1.

No such segregation was observed with pIJH7, and it was concluded that the Hae II-A fragment contained sufficient information to at least extend the survival of the pBR322 replicon in continuous culture.

However, it is prudent here to introduce a note of caution. It was shown convincingly in Results III that the lag period prior to the detection of pBR322 segregants was a function of the copy number of the plasmid; segregation of R- cells only occurred after a drop in copy number such that the probability of a daughter cell not receiving a plasmid copy was high. The copy numbers of pIJH1 and pIJH7 were not determined and although no increased yield of plasmid was observed when strains bearing these plasmids were lysed, it remains possible that both pIJH1 and pIJH7 exhibited an elevated copy number such that the segregation of R- cells did not occur within the length of the culture runs tested. Clearly further examination of these plasmids in continuous culture is warrented before the 'par' phenotype of the Hae II-A fragment can be substantiated.

Complete stabilization of the pBR322 replicon was observed when the 'par' function derived from pPM31 (and originally from pSC101) was cloned into pBR322; no segregants were ever detected in cultures of W5445 (pIJ005). This result confirms the observation of Meacock and Cohen (1980) that the 'par' function can stabilize another unrelated replicon pACYC.184. Moreover, since the 'par' function is small (370 bps) it suggests the utility of this fragment as a general means to stabilise unstable plasmids; the extra maintenence energy required to replicate an extra 370 bases of DNA is negligible.

The note of caution introduced earlier for pIJH1 and pIJH7 is also applicable to the results obtained with pIJ005. However, since no copy number or replication functions were ascribed to this fragment during its initial characterisation (Meacock and Cohen, 1980) it seems unlikely that increase in copy number could explain the increased stability of pIJ005 in continuous culture.

iv) Nature of the 'par' function and overall conclusions

The results presented in this thesis describe the analysis of plasmid stability using continuous culture techniques. Such an analysis has shown that two otherwise highly related plasmids pBR322 and pDS1109 exhibit altered properties of inheritable stability. To date, pDS1109 has failed to give rise to detectable levels of R- segregants and thus must be considered highly stable. By contrast pBR322 has been shown to give rise to R- segregants within 40 generations of continuous growth. The results strongly imply the existence on natural ColE1 type plasmids of a function actively involved in partitioning of plasmids between daughter cells; this function is evidently mutant or missing in pBR322.

In addition to the study of stability presented in this work, two other groups of workers have recently published similar findings. Wouters *et al*. (1980) also reported the loss of pBR322 in continuous culture under conditions of both phosphate and glucose limitation. Their results were obtained using an independently obtained stock of pBR322 and a different strain of *E. coli* as host, further indicating the independence of plasmid instability on host strain. An interesting additional finding of Wouters _et al_. was that the lag period prior to the detection of R- segregants could be prolonged by growth at lower temperatures (30°C) and shortened by growth at higher temperatures (42°C). By analogy with the results obtained in this thesis it is likely that growth at lower temperatures reflects less stringent conditions of nutrient limitation and thus slows the rate of plasmid copy number decline and prolongs the lag period before R- segregants are detected. Although not directly tested, a drop in the copy number of pBR322 prior to the detection of the R- strain was also suggested by Wouters _et al_.

In contrast however to the results reported by this thesis (see Jones _et al_., 1980) and those reported by Wouters _et al_. (1980), survival of pBR322 has been described in strains growing under glucose limitation in the chemostat (Noack _et al_., 1982). Under identical conditons however these authors report the loss of plasmid pBR325, a direct descendant of pBR322! pBR325 (Bolivar _et al_., 1977) contains all the genetic information of pBR322 and in addition the gene for chloramphenicol resistance inserted into a region of 'silent' DNA some 2 Kb away from the origin of replication. It is difficult to see why two such related plasmids should behave differently in continuous culture but, despite the homology shared by these plasmids, Noack _et al_. do not discuss the possible reasons for their altered partition phenotypes. Reference to the thorough study of plasmid copy number presented in this thesis may provide an explanation for the findings of Noack _et al_. An inverse relationship has been found between the size of some ColE1 derivatives and their copy number (Dougan and Sherratt, 1977), it is thus reasonable to suppose that the copy number of pBR325 is slightly lower than that of pBR322. It follows that, in continuous culture segregants of pBR325 will arise before those of pBR322. Since the conditions of growth employed by Noack *et al*. only detect segregants of pBR325, it may be that cultivation in stringent conditions of nutrient limitation was not continued long enough in cultures of pBR322 to ensure the detection of the R- population. An alternative possiblity is that the overall growth conditions themselves were not suitable to decrease the copy number of pBR322 and again ensure segregation. Neither of these possibilities were examined by Noack *et al*.

The observations on the stability of multicopy plasmids in these examples provide good evidence to imply the existence of a 'par' function for efficient plasmid segregation. 'Par' elements have also been implied in other plasmid systems. In addition to the multicopy ColE1 type plasmids, a multicopy *Staphylococcal* plasmid (pT169) has been shown to be equipartitioned under conditions of low copy number (implying a 'par' function) (Della-Latta *et al*., 1978) and the multicopy *E. coli* plasmid pACYC184 has been shown to segregate plasmid free cells in the absence of selective pressure (Meacock and Cohen, 1980).

As instability of low copy number plasmids cannot be masked by high copy numbers, the number of reports of putative 'par' elements amongst such plasmids is higher. In particular, many groups have reported instability for mutants of the large low copy number plasmids of the inc FII incompatability group (R1, Nordstrom *et al*., 1980; NR1, Miki *et al*., 1980; R6-5, Timmis *et al*., 1981; RMS201, Ike *et al*., 1981) and in addition, *par* mutants have been described for the plasmid P1 (Scott *et al*., 1978; Sternberg and Austin, 1981) and for derivatives of the F factor (Lane, 1981; Bergquist *et al.*, 1981).

It is therefore apparent that many, perhaps all naturally occurring plasmids possess mechanisms for their efficient segregation into daughter cells at division.

How could such a mechanism operate?

The number and variety of plasmids that appear to possess a 'par' function suggests the likelihood of there being more than one form of segregational mechanism but despite this fact, the final interaction that accomplishes segregation is likely to be similar. Efficient segregation can only occur if the separation of plasmid molecules is 'tied' to some degree to the act of cell division. To achieve this, it is likely that plasmids are linked at least transiently to either the cell membrane or the bacterial genome, presumably in some nucleoid type structure. Some plasmids, notably the *E. coli* F factor can be isolated together with the folded *E. coli* chromosome implying a linkage that could ensure segregation ( Kline *et al.*, 1976), however, as the *E. coli* chromsome itself can be isolated bound to membrane (Worcel and Burgi, 1972) it is perhaps more likely that membrane attachment of plasmids ensures their segregation. There are cases where the coupling of plasmid replication to cell division appears very strong; mutants of the plasmid R1 for example cause failure of cell division in a significant proportion of their hosts (Engberg *et al.*, 1975) and there is evidence for a link between certain plasmid rounds of replications and subsequent cell division (Rosner *et al.*, 1968).

Recently, a site for the binding of the _E. coli_ chromosome has been determined close to the origin of replication (Wolf-Watz and Norquist, 1979; Wolf-Watz and Masters, 1979; Nagai _et al._, 1980), and it is possible that 'par' loci represent similar membrane binding sites for plasmid molecules. In this respect it should be noted that Gustafsson _et al._ (1983) have described the ability of the 'par' locus from pSC101 (Meacock and Cohen, 1980; and see Chapter V) to confer upon plasmids the ability to bind to the outer membrane of _E. coli_. A region implied as containing a 'par' locus from the plasmid R1 (inc FII) also permitted membrane binding. These results imply that at least for plasmids pSC101 and R1 partitioning of molecules at division involves binding to the outer membrane of _E. coli_.

For small plasmids such as pSC101, it is likely that membrane binding is the only prerequisite for segregation for the 'par' locus has been shown to only act _in cis_. Moreover, since the 'par' function has been localized to only 270 bps of DNA it is unlikely to involve encoded proteins. This does not however infer that all partition mechanisms act _in cis_, the inc FII plasmid RMS201 appears to have at least two 'par' sites (Ike _et al._, 1981) and the F factor may have three loci involved in partitioning (Ogura and Hiraga, 1983).

The results presented in this thesis and confirmed by Wouters _et al._ (1980) suggested that multicopy plasmids also contain an efficient 'par' function , and the results obtained with _trans_ acting plasmids present in the same cell as the _par_ defective pBR322 suggest this function also acts only _in cis_. This suggestion is supported by the localization of the putative 'par' element to the Hae II-A fragment of pDS1109, a region of DNA that has been shown to encode the Colicin E1 immunity protein but for which no other proteins have been found (Dougan and Sherratt, 1977; Collins, 1978). In agreement with all other par elements described, the putative 'par' element mapped to pDS1109 but not pBR322 by this work is evidently separate from plasmid replication although it should be noted that the region involved in partitioning does lie near the presumptive terminus of replication, a position where one might expect a 'par' function to act.

It is unlikely that the 'par' function operates on all plasmids at the same time, under conditions of low growth rate very few of the pool of plasmids within a cell will be undergoing replication. Moreover, despite the par- phenotype of pBR322, no segregants were detected during the first 30-40 generations of growth even though they were sure to takeover the culture had they arisen. These results suggest that although actively replicating molecules may be membrane bound and ensured of segregation, the majority of the population of plasmids is passively inherited by random distribution. A conclusions that is supported by a model based on theoretical considerations recently proposed by Hashimoto-Gotoh and Ishii (1983).

v) Final Conclusions

A good deal of progress has been made during the experiments presented here on the existence and nature of partition elements amongst multicopy plasmids.

Natural ColE1 type molecules (pDS1109) have been shown to segregate as efficiently into daughter cells as their large low copy number counterparts such as RP4 and RP1. The efficiency of segregation observed for pDS1109 did not hold true of two constructed ColE1 relatives, pBR322 and pMB9, suggesting the loss of the 'par' element during their construction.

By experimentation and argument it was shown that the probable area of pDS1109 involved in segregation lay upstream of the origin of replication and that its presence *in cis* on pBR322 did indeed stabilize its normal defective partitioning.

A known 'par' element from the plasmid pSC101 was also shown to stabilize pBR322 confirming its ability to act heterospecifically. Finally, the mode of action of such 'par' elements has been considered and compared to those implied or known to exist on other plasmids.

The conclusions reached by this work may signify a note of caution for those interested in the commercial exploitation of recombinant DNA technology. If a bacterial strain containing a plasmid based on pBR322 is grown in continuous culture two problems may arise. Firstly, there may be a drop in product yield concomitant with a drop in plasmid copy number and secondly, any plasmid free segregant arising could quickly come to dominate the culture. Moreover, although these results have been obtained using continuous culture, similar findings might be expected in large batch culture fermenters were the inoculum is prepared by a stepwise sequence of growth involving increasing amounts of media.

Further research of plasmid carrying populations in the chemostat is clearly required if proposals for the correction of these defects are to be forwarded.

## Results and Discussion - II

# VI The effect of growth rate and nutrient limitation on the plasmid mediated transformation of *E. coli* W5445

i) Introduction to *E. coli* transformation

ii) Experimental Procedures

iii) Dose Response curve for W5445 and pBR322 DNA

iv) Culture pH and transformation frequency

v) Effect of growth rate and nutrient limitation on the frequency of transformation

vi) Sudden growth rate changes (shift-up) and the transformation frequency

vii) Influence of cell size alone on transformability

viii) Shift-up is followed by return to steady state

ix) $Mg^{2+}$ and Sulphur limited cultures and the efficiency of transformaton

x) Growth rate and the efficiency of transformation

i) Introduction to E. coli transformation

Throughout the course of the experiments presented in the preceeding chapters on plasmid stability, a phenomenon repeatedly encountered was that of persistence of an uncompetitive strain at low levels of the population in the chemostat. It had already been shown (cf. Results Chapter I) that persistence could not be explained simply as an artefact of the culture system such as wall growth or as a consequence of conjugation within the culture media. Nevertheless, the ubiquity of low level persistence in competitive situations had led some authors (see Godwin and Slater, 1979) to suggest that persistence was the manifestation of some as yet unexamined form of genetic exchange in the chemostat. Indeed such a mechanism might be considered advantageous if one considers the possibility that maintenence of an otherwise non-useful piece of genetic information might ensure the survival of the strain at some future date when the marker carried at low levels in the population is selected for.

Any form of genetic exchange that occurs within a chemostat culture must be able to contend with the normal culture conditions; the high shearing rates in the culture present to ensure good aeration also ensures that cell to cell contact is only a transient event. Any form of genetic exchange requiring such contact can therefore be assumed not to occur and exchange must be mediated by some external agent such as a bacteriophage (transduction) or naked DNA (transformation).

As no bacteriophage are present within normal, aseptically run continuous culture systems, direct transformation could, at least in theory, be able to occur within the chemostat population. In order to examine this possibility and because no detailed analysis of the ability of DNA to transform chemostat grown cells had been done, experiments were designed to examine the effect of growth rate and nutrient limitation on the transformability of E. coli with plasmid DNA.

Transformation, the process whereby naked DNA enters a living cell and becomes established, is a well documented system of genetic exchange for several bacterial species. Experimental systems based on the two Gram positive species Bacillus and Streptococcus have led to the understanding that the ability of an organism to take up DNA, a state referred to as competence occurs only during certain phases of the bacterial growth cycle. For example, Bacillus subtilis becomes competent for DNA uptake for a relatively short period prior to the onset of stationary phase (Venema, 1979).

The establishment of competence in both the well characterised systems of Bacillus and Streptococcus spp. is a natural event; competent cells appear spontaneously at certain stages of growth and no specific treatment is required to induce their appearance. This is not the case for all organisms and in these cases competence is 'induced' by a variety of chemical treatments.

Competence in E. coli for example is unveiled by the pretreatment of the cell population with a solution of divalent cations, the most efficient of which is calcium (as $CaCl_2$). This original observation made in 1970 by Mandel and Higa (1970) using purified phage DNA as the transforming agent was subsequently shown to hold true when the transforming agent was plasmid DNA (Cohen et al., 1972) or chromosomal DNA (Oishi and Colsoy, 1972).

Pretreatment of recipient cells with $Ca^{2+}$ ions has also been shown to induce competence in a variety of other organisms spanning Gram positive and Gram negative species. (*Klebsiella aerogenies*, Taketo, 1972; *Salmonella typhiphurium*, Lederberg and Cohen, 1974; *Pseudomonas putida*, Chakravarty *et al*., 1975; *Mycobacterium smegmatis*, Norgard and Imeda, 1978; and *Staphylococcus aerus*, Rudin *et al*., 1974). The extent to which this diversity may indicate a common mechamism for $Ca^{2+}$ ion action is not known.

Optimization of the factors within the experimental procedures available for the establishment of competent *E. coli* cells have been reported by several groups and although very similar, the conditions optimal for transformation by chromosomal DNA are different from those optimal for plasmid DNA (see Kushner ., 1978; Humphreys *et al*., 1979; Dagert and Ehrlich, 1979; Bergmans *et al*., 1981) an observation that may indicate differences in the uptake of each type of molecule by the cell.

For plasmid DNA, the procedure widely adopted for the generation of competent cells is based on the method reported by Cohen *et al*. (1972). An exponential culture of *E. coli* is harvested and washed in a cold solution by $CaCl_2$ (usually 30 mM). After a short incubation on ice the cells are repelleted in the cold and finally taken up in a small volume (usually 1/20th-1/50th of the original culture volume) of 75-100 mM calcium chloride. The final cell suspension is stored on ice and is 'competent' for several days. The transformation procedure itself has also been optimised for times and temperatures of incubation and is usually as follows: a sample of DNA is added to the cell suspension on ice and the mixture maintained at $0^{o}C$ for up to 45 minutes. After this period of incubation the cell/DNA mixture is transferred to a water bath at 37°C or 42°C (depending on protocol) and 'heat shocked' for about 5 minutes. During this time DNA bound to the cell surface in the cold incubation step becomes resistant to the action of DNAase and is thus regarded as intracellular. Cell populations transformed in this way are then either plated directly with selection of the transformed marker or allowed a period of recovery in non-selective media before selection.

Using conditions for the transformation protocol standardized in this way, several groups have analysed the levels of competence achieved when E. coli is harvested at different times in the bacterial growth cycle.

The transformation efficiency of E. coli was shown to increase dramatically after dilution of stationary phase cultures into fresh media (Oishi and Irbe, 1977), and the relationship between growth phase and transformability was systematically examined by Brown et al. (1979) who found peak of transformation efficiency for plasmid DNA as E. coli C600 entered the exponential phase of growth. Despite the fact that many transformation systems require a competence factor released into the media to elicit high transformation levels, Brown et al. could find no evidence of any such factor in the culture media of E. coli populations and concluded the sharp peak of transformation to be a consequence of unique growth rate changes at this stage of the culture life. A similar finding was made by Norga rd et al. (1978) with the E. coli strain X1776 suggesting it as a general feature of E. coli transformation.

Despite the optimization of both the phase of growth chosen for culture harvesting and the parameters of the transformation procedure itself, only 0.1%-1% of the surviving population after $CaCl_2$ treatment become transformed (Humphreys et al., 1978). When chromosomal DNA is the transforming agent the maximum frequency attainable is even lower at between $10^{-5}$ and $10^{-4}$ per viable cell (Bergmans et al., 1981).

Since greater than 99% of the $CaCl_2$ treated populations do not become transformed in any one experiment, conclusions drawn about the action of $Ca^{2+}$ ions on the population are limited. Nevertheless, those reports that do suggest a mechanism of transformation indicate a role for $Ca^{2+}$ ions in the alteration of the cell wall structures of E. coli.

The ability of E. coli spheroplasts to become transformed in the absence of the usual high levels of $CaCl_2$ suggests a role for this cation in altering the permeability of the cell wall (Henner et al., 1973; Mo̊lholt and Doskocil, 1978). This supposition is supported to some extent by the finding that alteration of the lipopolysaccaride moiety of the cell wall of a closely related Gram negative organism, Salmonella typhimumum stimulated its ability to be transfected with phage DNA (Burstyn et al., 1975).

A direct role for $Ca^{2+}$ ions in the binding of DNA to the cell walls of E. coli has been reported by Sabelnikov et al. (1975) and more recently this observation has been thoroughly explored by Weston et al. (1981). Their results suggest that not only does $Ca^{2+}$ stimulate binding of DNA to outer membrane fractions of E. coli, but that the efficiency with which it does so in relation to other divalent cations parallels exactly their relative efficiency in inducing competence. The general proteolytic enzyme trypsin destroyed the ability of isolated membranes to bind DNA in the presence of $Ca^{2+}$ ions thus suggesting a role for some protein(s) in the act of DNA binding.

Using conditions optimized for the uptake of chromosomal DNA, Bergmans et al. (1981) have shown that leakage of certain periplasmic enzymes into the transformation mixture is associated with the state of competence, the higher the leakage the higher the transformation efficiency observed. These authors conclude that $Ca^{2+}$ ions disrupt to some degree the outer membrane of E. coli thus allowing any transforming DNA present to bind to receptors in the periplasm or the inner membrane.

Based on these and other findings, several schemes of DNA uptake in E. coli have been suggested (see Humphreys et al., 1979) but the proposal of a more definitive mechanism must wait for technological advances that allow the enrichment of the subpopulation of transformable cells.

One problem in the study of competent E. coli populations is the method of growth prior to their production. As already noted in an earlier section the efficiency of transformation observed varies dramatically throughout the growth cycle, the most susceptible cells being those in the early exponential phase. However, even within this one limited phase of growth in batch culture, the growth rate of individual cells is constantly changing, moreover, the identity of the factors controlling growth at that time are seldom known. Such heterogeneity amongst the population cannot help but confuse any conclusion drawn about the stage of cell growth and the susceptibility to transformation.

In the light of these considerations we sought to investigate the effect of precisely controlled environmental factors such as growth rate and nutrient limitation on the transformability of E. coli by plasmid DNA. Moreover, we were intrigued by the possibility that, by cultivating cells under precisely controlled growth conditions we might be able to either increase the maximum efficiency of transformation so far observed or discover conditions under which E. coli exhibited natural competence and would take up exogenous DNA without pretreatment with $Ca^{2+}$ ions.

Plasmid DNA was chosen as the transforming agent for all experiments for its ease of preparation and the fact that it represents a pure population of one selectable marker. In addition, the necessity for recombination with the host genome as a prerequisite for replication and maintenence as is the case for chromosomal DNA is absent in the case of plasmid DNA.

ii) Experimental Procedures

The plasmid chosen as the source of transforming DNA was pBR322, the ColE1 related cloning vector much used in earlier work of this thesis. pBR322 DNA was purified from cleared lysates by chromatography on hydroxyapatite and stored at -20°C prior to its use in transformation experiments. The concentration of DNA was assessed from the absorbance at 260 nm (1 OD unit = 50 μg.ml$^{-1}$ DNA).

The physical integrity of pBR322 DNA so prepared was assessed by agarose gel electrophoresis, a typical preparation was in the main (≈70%) covalently closed monomeric circles with some evidence of dimeric circles and relaxed (nicked) monomers. Preparations were essentially free of linear DNA which does not transform E. coli

The recipient E. coli strain for all transformation experiments was E. coli W5445, a restrictionless derivative of E. coli K12 (Backman, 1972), in common use as a transformation recipient in the laboratory.

Chemostats were run under the nutrient limiting protocols described in Materials and Methods and allowed to equilibriate at any new steady state for at least 10 generations.

Samples of chemostat grown cells were taken and stored at -20°C in 15% (v/v) final concentration of glycerol prior to the transformation event itself. In reconstruction experiments this was found not to alter the observed transformation efficiency when compared to cells transformed directly upon removal from the chemostat. Cryogenic preservation of competent cells has also been reported by Morrison (1979).

The transformation step itself was that described by Humphreys et al. (1979) and described fully in Materials and Methods. Briefly, accumulated samples of chemostat grown cells were thawed and made competent by resuspension in 75 mM $CaCl_2$. All samples were then transformed by aliquots of the same DNA solution. After the heat shock, samples were diluted with an equal volume of L-broth and held at 37°C for 45 minutes prior to plating for the selection of transformants.

Efficiencies of transformation are expressed as the numbers of transformants per viable cell at the time of plating.

iii) Dose response curve for W5445 and pBR322 DNA

The aim of these experiments was to examine the relationship between different culture variables, in particular growth rate and nutrient limitation, and the numbers of transformants obtained after transformation.

It was thus essential that in all experiments the DNA present in all transformation mixes was not limiting. To control for this possibility a dose-response curve was produced for the transformation of E. coli W5445 with pBR322 DNA.

Cells for this experiment were grown in batch culture and harvested at times suggested by Brown et al. (1978). They were rendered competent and transformed with increasing amounts of plasmid DNA. The curve of response so obtained is shown in figure 23. Saturation occurred in a standard aliquot of competent cells (0.1 ml) with about 250 ng of plasmid DNA (i.e. 2.5 $\mu g.ml^{-1}$). This concentration of DNA gave a transformation frequency of $5 \times 10^{-3}$ per viable cell, a number similar to that reported by Brown et al. for cells harvested in early exponential phase.

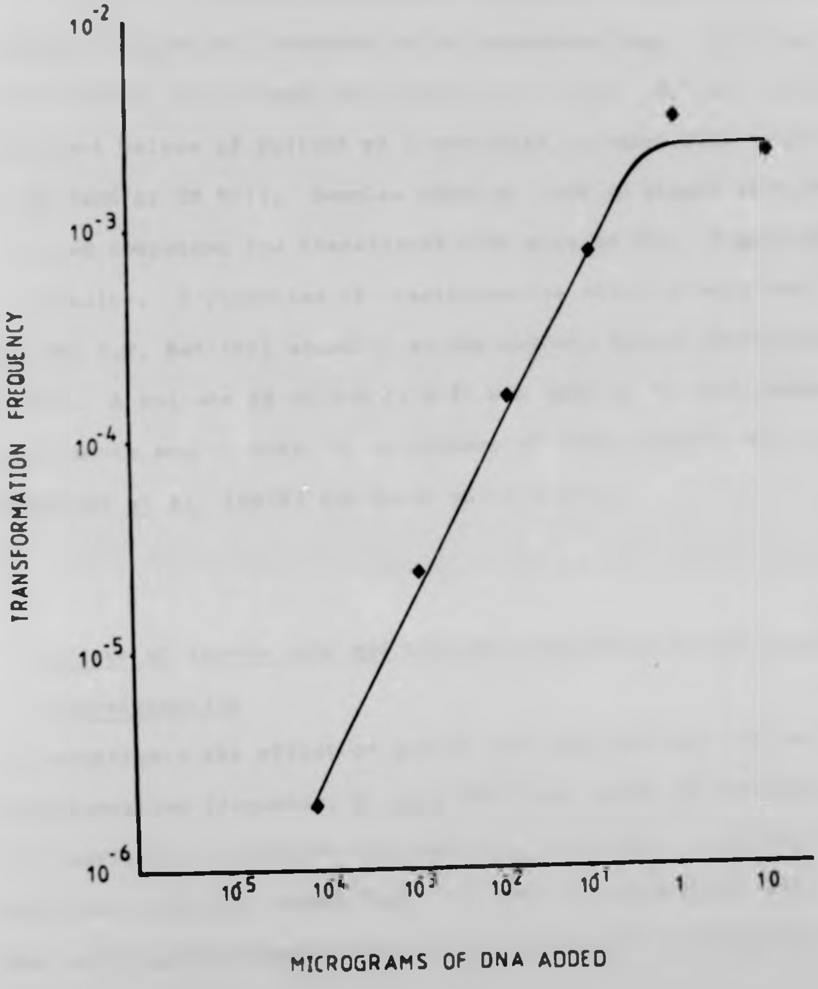
To ensure saturation in all subsequent experiments a concentration of five times the saturating value was adopted for each transformation mix (i.e. 12.5 $\mu g.ml$).

iv) Culture pH and transformation frequency

Before embarking on an examination of culture variables and

Figure 23. A dose response curve for the transformation of *E.coli* W5445 with pBR322 DNA.

Varying amounts of pure plasmid DNA were added to a standard aliquot of *E.coli* W5445 prepared for transformation as described in the text. After the transformation protocol, the transformation mix was plated on both selective and non-selective plates. The transformation frequency (transformants per viable cell) was recorded after overnight incubation at 37°C.



TRANSFORMATION FREQUENCY
$10^{-2}$
$10^{-3}$
$10^{-4}$
$10^{-5}$
$10^{-6}$
$10^{-5}$
$10^{-4}$
$10^{-3}$
$10^{-2}$
$10^{-1}$
1
10
MICROGRAMS OF DNA ADDED

transformation, control experiments were done to ensure that the normal chemostat pH did not interfere with transformations. Cells were grown in 5% L-broth at a growth rate of 0.25 $hr^{-1}$ (Td  2.5 hr) at four different values of culture pH (controlled automatically by the addition of 2N NaOH or 2N HCl). Samples taken at each pH steady state were rendered competent and transformed with plasmid DNA. Figure 24 shows the results. Frequencies of transformation observed were similar at pH 6.0 and 7.0, but fell steadily as the culture became increasingly alkali. A culture pH of 6.8 (+ 0.2) was adopted for all subsequent experiments and we note the accordance of these results with those of Humphreys *et al*. (1979) for batch grown cells.

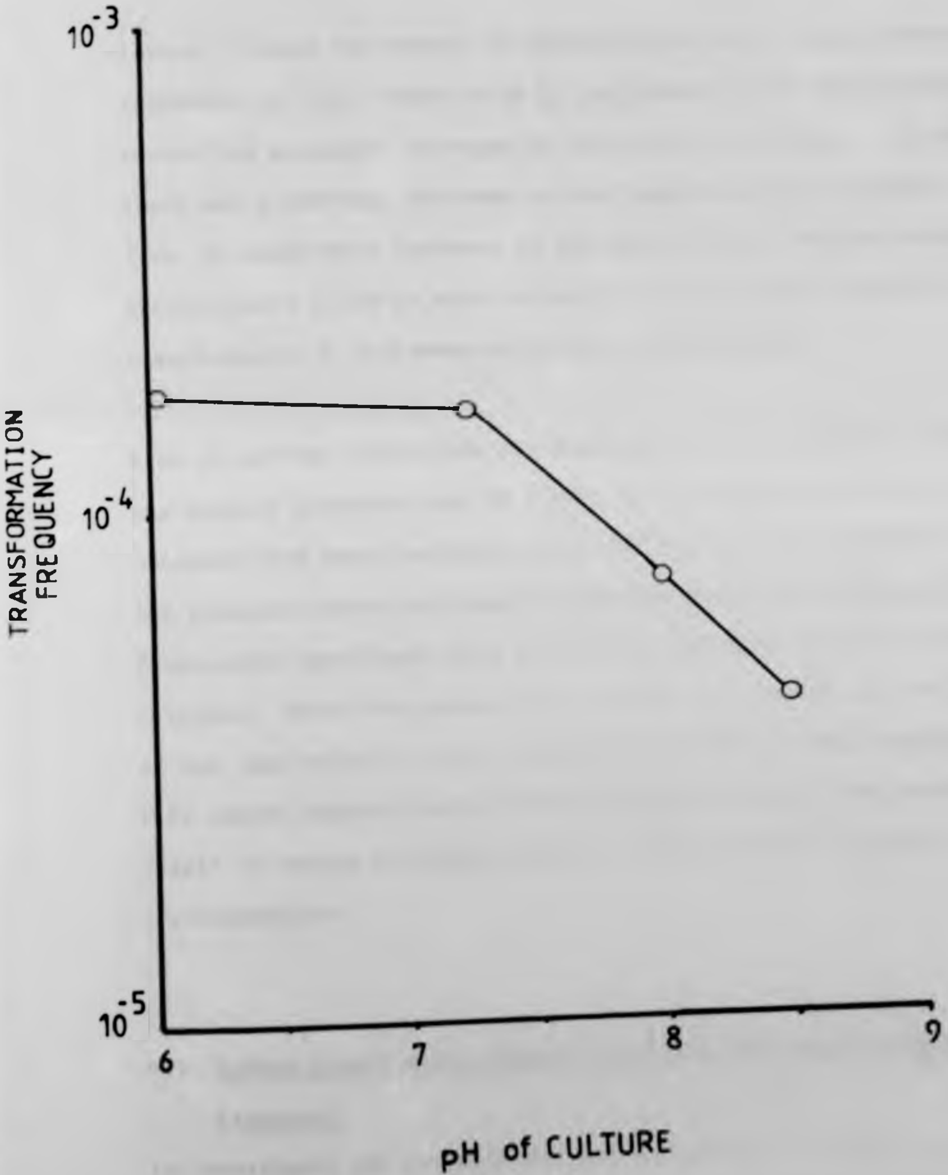
v) Effect of growth rate and nutrient limitation on the frequency of transformation

To investigate the effect of growth rate and nutrient limitation on transformation frequency, *E. coli* W5445 was grown in chemostat culture at a variety of different dilution rates and under a variety of different nutrient limitations. At least 10 generations were allowed for the re-establishment of a steady state prior to sampling.

Two samples were taken at each dilution rate and the final value of the transformation frequency was the average of both samples. At the end of each experimental run all samples were washed and transformed simultaneously. Because each cell sample was taken from cultures at the steady state (within each individual nutrient limitation) the proportions of transformable cells in each population were directly comparable. Moreover, reproducibility between experiments was good

<u>Figure 24.</u> The effect of culture pH on the steady state levels of transformation frequency.

A culture of <u>E.coli</u> #5445, maintained at a dilution rate of 0·25 hrs$^{-I}$ in 5 % L-broth (glucose limitation), was grown at a variety of pH values. At each steady state pH value samples of the culture were removed, made competent and transformed with saturating amounts of pBR322 DNA as described. The recorded transformation frequencies are the average of duplicate experiments.


TRANSFORMATION FREQUENCY
$10^{-3}$
$10^{-4}$
$10^{-5}$
6
7
8
9
pH of CULTURE

allowing a direct comparison between levels of transformation obtained at the same dilution rate (growth rate) but using different growth-limiting substrates.

Figure 25 shows the effect of different bacterial growth rates on the frequency of transformation of E. coli W5445 grown under conditions of carbon (as glucose), nitrogen or phosphorus limitation. In each case there was a 100-fold increase in the transformation frequency with a five- to eight-fold increase in the growth rate. Despite several attempts with cells at each dilution rate it proved impossible to obtain transformants in the absence of $CaCl_2$ pretreatment.

With phosphorus limitation the frequency of transformation obtained at the highest dilution rate (D = 0.8, Td = 50 mins) was close to that obtained with batch culture cells (cf. Fig.23), but with both nitrogen and glucose-limited cultures the maximum observed transformation frequencies were lower than either the phosphate limited or batch grown cultures. Since the growth rates of any cell population are identical at the same dilution rate irrespective of the nutrient limiting growth, this result implies that at least one factor other than growth rate itself is active in determining the final observed frequency of transformation.

vi) Sudden growth rate changes (shift-up) and the transformation frequency

To investigate the relationship between growth rate and transformability more precisely, the transformation response of E. coli W5445 was allowing a direct comparison between levels of transformation obtained at the same dilution rate (growth rate) but using different growth-limiting substrates.

Figure 25 shows the effect of different bacterial growth rates on the frequency of transformation of *E. coli* W5445 grown under conditions of carbon (as glucose), nitrogen or phosphorus limitation. In each case there was a 100-fold increase in the transformation frequency with a five- to eight-fold increase in the growth rate. Despite several attempts with cells at each dilution rate it proved impossible to obtain transformants in the absence of $CaCl_2$ pretreatment.

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vi) Sudden growth rate changes (shift-up) and the transformation frequency

To investigate the relationship between growth rate and transformability more precisely, the transformation response of *E. coli* W5445 was

<u>Figure 25.</u> Variation of transformation frequency in response to changes in growth rate and nutrient limitation.

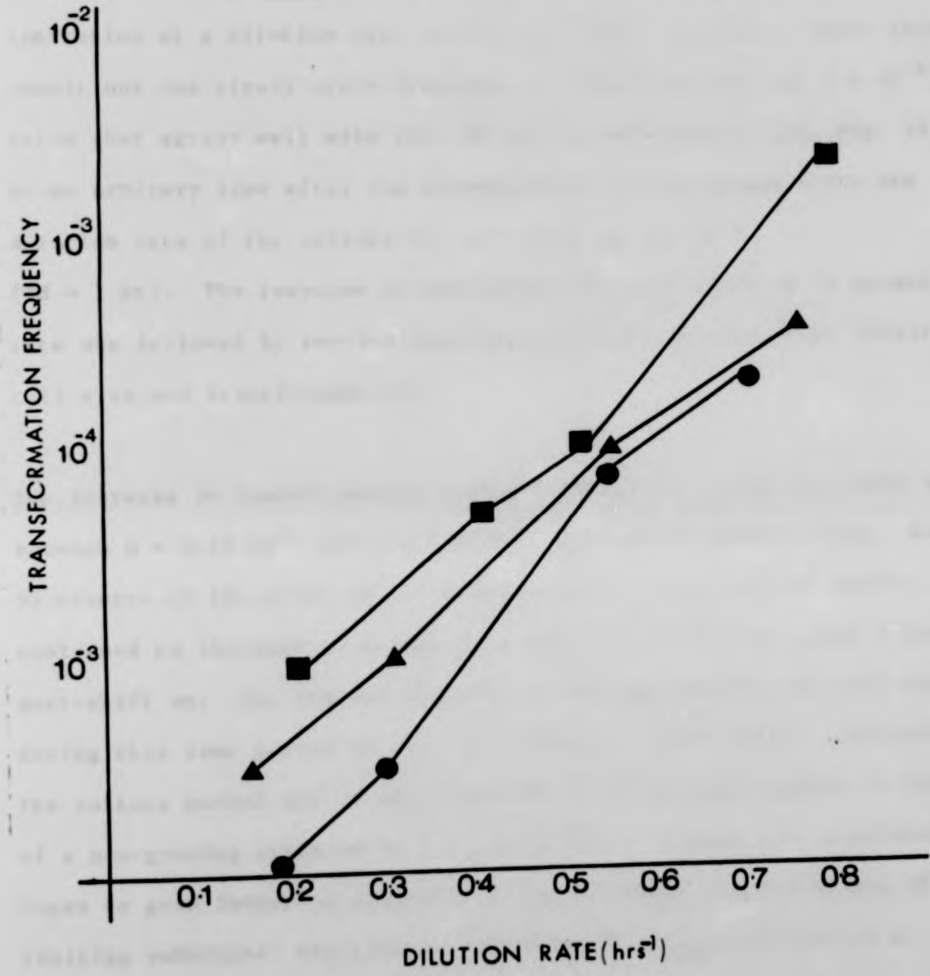
*E.coli* W5445 was grown at a variety of dilution rates under three different nutrient limitations. At each steady state growth rate, samples of the culture were removed, stored and transformed as described in the text. The final transformation frequency shown is the average of two platings.

The nutrient limitations employed were:

■ ---- phosphate limitation.

▲ ---- glucose limitation.

● ---- nitrogen limitation.



$10^{-2}$
$10^{-3}$
$10^{-4}$
$10^{-3}$
TRANSFCRMATION FREQUENCY
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
DILUTION RATE(hrs$^{-1}$)

followed during the transition from one steady state to another. For this experiment E. coli W5445 was grown under conditions of phosphate limitation at a dilution rate of 0.15 $hr^{-1}$ (Td = 4.5 hr). Under these conditions the steady state frequency of transformation was $7 \times 10^{-6}$, a value that agrees well with that of earlier experiments (cf. Fig. 25). At an arbitary time after the establishment of the steady state the dilution rate of the culture was increased to 0.6 $hr^{-1}$ (Td = 1 hr). The response of the culture to this shift up in growth rate was followed by regular sampling and analysis of optical density, cell size and transformability.

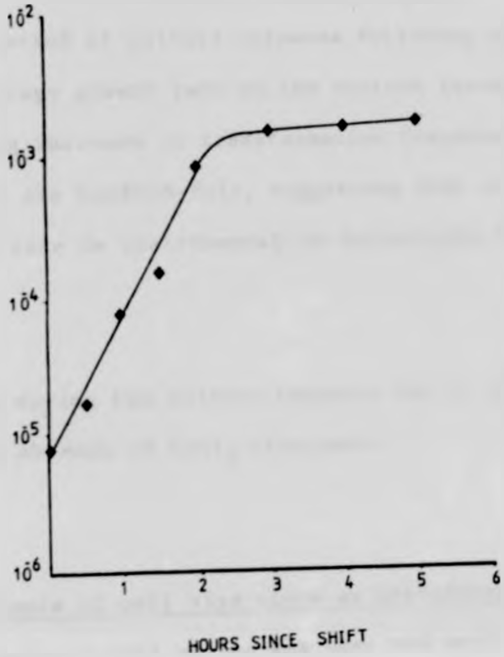
The increase in transformation frequency observed during the shift up between D = 0.15 $hr^{-1}$ and D = 0.6 $hr^{-1}$ is shown in Figure 26(a). Within 30 minutes of the shift up the transformation frequency had doubled, it continued to increase to a peak frequency of $2 \times 10^{-3}$ at about 3 hours post-shift up. The changes observed in optical density and cell volume during this same period of time are shown in Figure 26(b). Initially the culture washed out of the chemostat with kinetics similar to those of a non-growing organism at D = 0.6 $hr^{-1}$ but, as the cell population began to grow faster in response to the increased concentrations of limiting substrate, the rate of reduction in $OD_{540}$ decreased to a plateau at 4 hr post-shift up.

The changes in cell volume observed from Coulter Counter analysis of each time point showed an increase in size that paralleled the increase in growth rate. The relative cell volume reached a peak of double its original value at 4 hr post-shift up, when growth rate was at a maximum.

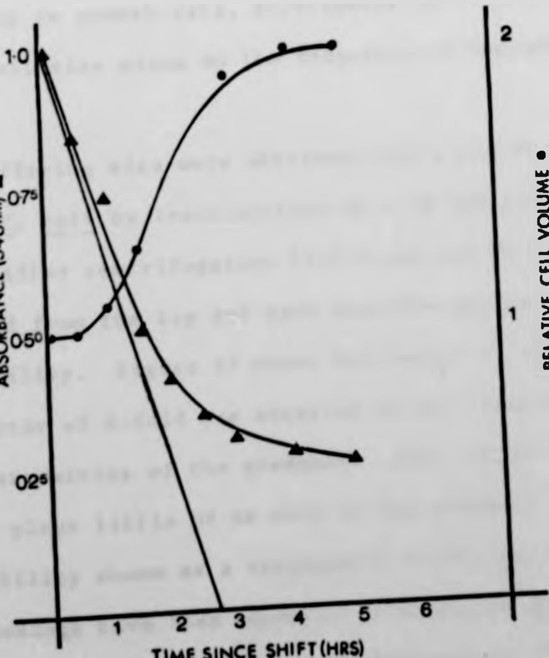
Figure 26. Changes in transformation frequency following a shift-up in culture dilution rate.

E.coli #5445 was grown in a phosphate limited chemostat, and at time zero the dilution rate was increased from 0·15 $hrs^{-1}$ to 0·6 $hrs^{-1}$. Following this shift-up in growth rate, the culture was sampled regularly and the changes in transformation frequency (fig. 26(a)) were compared with changes in culture optical density (▲) and relative cell volume (●) (fig. 26(b)). The theoretical washout rate for a dilution rate of 0·6 is also shown (—).

(a)


TRANSFORMATION FREQUENCY
HOURS SINCE SHIFT

(b)


ABSORBANCE (540nm) ▲
RELATIVE CELL VOLUME ●
TIME SINCE SHIFT (HRS)

During the period of culture response following the shift up in dilution rate the average growth rate of the culture increased by four-fold. The corresponding increase in transformation frequency over this period was greater than one hundred-fold, suggesting that at least one factor other than growth rate is instrumental in determining final transformation numbers.

At no point during the culture response was it possible to transform *E. coli* in the absence of $CaCl_2$ treatment.

vii) Influence of cell size alone on transformability

As an increase in cell volume was observed during the culture response to a shift up in growth rate, experiments were done to determine the effect of cell size alone on the frequency of transformation.

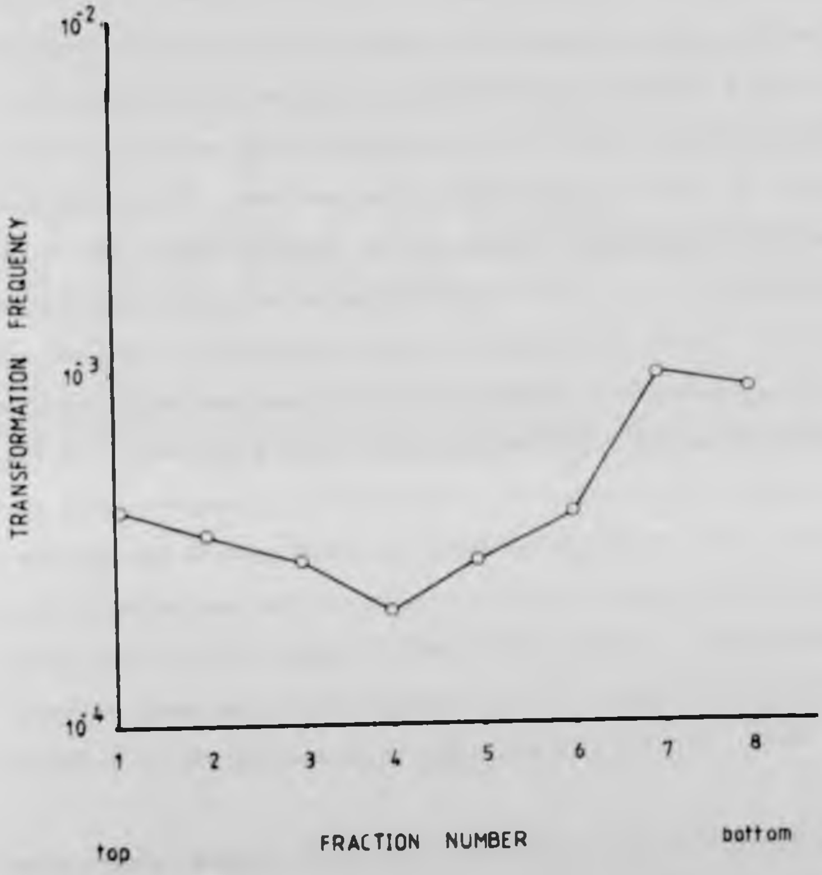
Cells of differing size were obtained from a mid-exponential phase culture of *E. coli* by fractionation on a 5%-20% continuous sucrose gradient. After centrifugation (3.5 K rpm for 20 min) the gradient was fractionated from the top and each fraction analysed for cell size and transformability. Figure 27 shows the result of such an analysis, a maximum factor of 4-fold was observed in the transformability of cells from the extremities of the gradient. This result suggests that cell size alone plays little or no role in the dramatic increases in transformability shown as a response to shifts up in growth rate. Similar findings have been reported by Humphreys *et al*. (1979) who failed to observe any increase in transformation frequency after fractionation on gradients of metrizamide.

Figure 27. The transformation frequency of E.coli W5445 separated into size classes by sucrose gradient centrifugation.

A batch grown culture of E.coli W5445 was taken and treated as described in the text.
After centrifugation, the gradient was fractionated from the top and each fraction made competent and transformed. Transformation frequencies so obtained were plotted against fraction number.

__Figure 27.__ The transformation frequency of _E.coli_ W5445 separated into size classes by sucrose gradient centrifugation.

A batch grown culture of _E.coli_ W5445 was taken and treated as described in the text.
After centrifugation, the gradient was fractionated from the top and each fraction made competent and transformed. Transformation frequencies so obtained were plotted against fraction number.



$10^{-2}$
$10^{-3}$
$10^{-4}$
TRANSFORMATION FREQUENCY
1
2
3
4
5
6
7
8
top
FRACTION NUMBER
bottom

In addition to the analysis of each cell-size fraction described above, a sample of the smallest cells (representing those cells that had just divided) was inoculated into pre-warmed nutrient broth and immediately agitated vigorously to encourage rapid growth. Such a culture is 'synchronized' with respect to growth for the subsequent two or three waves of division (Mitcheson and Vincent, 1965). Transformability was followed through these cycles of division and the result is shown in Figure 29. Synchrony held for two complete doublings of the population which subsequently became heterogenous with respect to growth phase. Despite the well separated waves of division observed, the frequency of transformation remained relatively constant throughout the monitored period. There was a small though repeatable stimulation in frequency just prior to each cycle of divison. The observation suggests a limited number of DNA binding sites for transformation per cell; just prior to cell division each cell is twice its normal length and therefore possesses twice the number of DNA binding sites per colony forming unit. A similar observation has been made for the number of bacteriophage receptors on the surface of _E. coli_ (Onken and Messer, 1973).

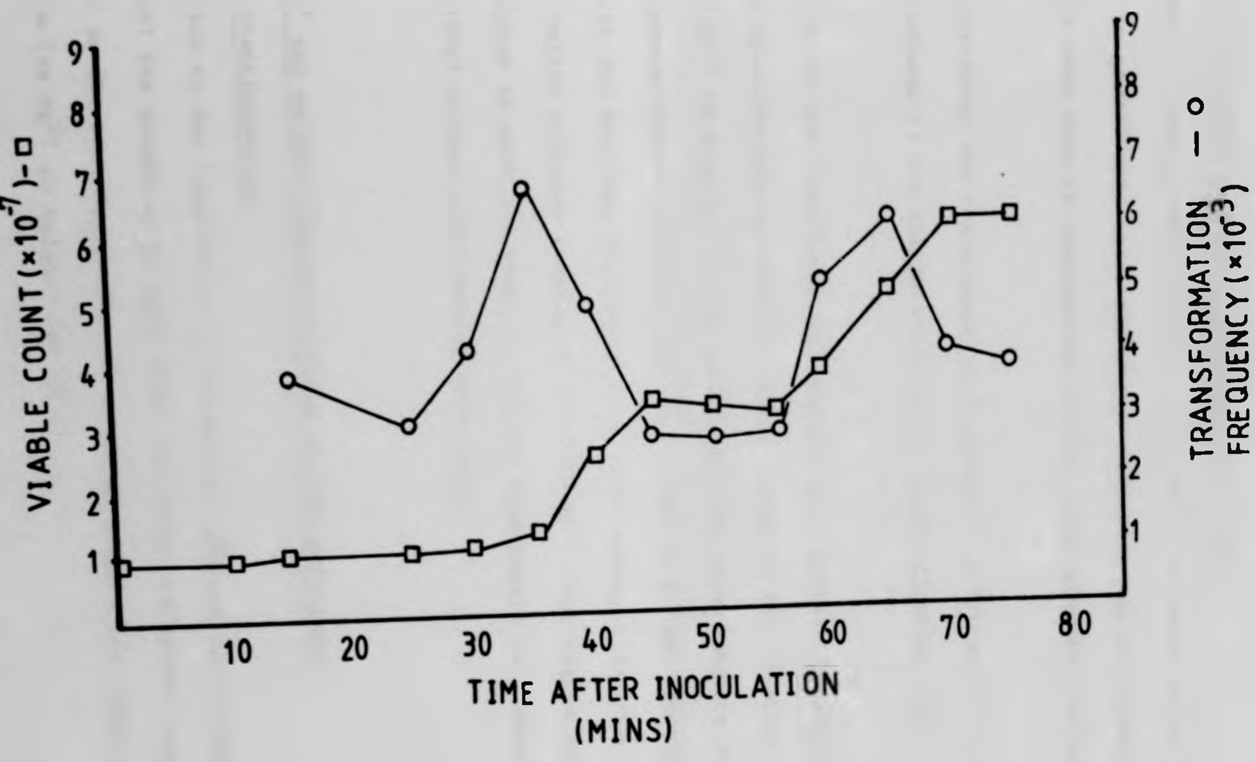
These results suggest that cell size alone plays little part in the increase in transformation frequency observed following a shift up in growth rate, cells of larger than unit length may be marginally more transformable than those of unit length, but this is largely a consequence of their increased surface area available for DNA binding rather than an alteration in the case of the transformation event per cell.

**Figure 28.** Changes in transformation frequency throughout synchronised division of *E.coli* W5445.

A top fraction from a sucrose gradient separation of a culture of *E.coli* W5445 was inoculated into fresh prewarmed L-broth and incubated with vigorous aeration at 37°C.

Samples taken throughout the subsequent incubation period were plated for viable count and transformed in the usual manner.

The changes in transformation frequency ( O ) and viable count ( □ ) were plotted against the age of the culture.



VIABLE COUNT (×10⁻⁷) - □
TRANSFORMATION FREQUENCY (×10⁻³) — o
TIME AFTER INOCULATION (MINS)

viii) Shift up is followed by return to steady state

The increase in transformability of E. coli W5445 observed during the shift up in growth rate under phosphate limitation was also observed when shifts were done on populations growing under glucose limitation.

In this experiment the transformation frequency was followed into the re-establishment of the steady state (at a higher dilution rate).

Figure 29 shows the transformation response of a culture throughout a period of re-establishment after a sudden change in the dilution rate from 0.25 $hr^{-1}$ to 0.55 $hr^{-1}$. In accordance with those results obtained from phosphate-limited cultures the e.o.t. rose to a peak several hours after shift and declined thereafter to a level normally observed for glucose-limited cultures growing at D = 0.55 $hr^{-1}$ (see Figure 25). With these samples as with all those in earlier experiments no transformants were obtained without pretreatment with $CaCl_2$.

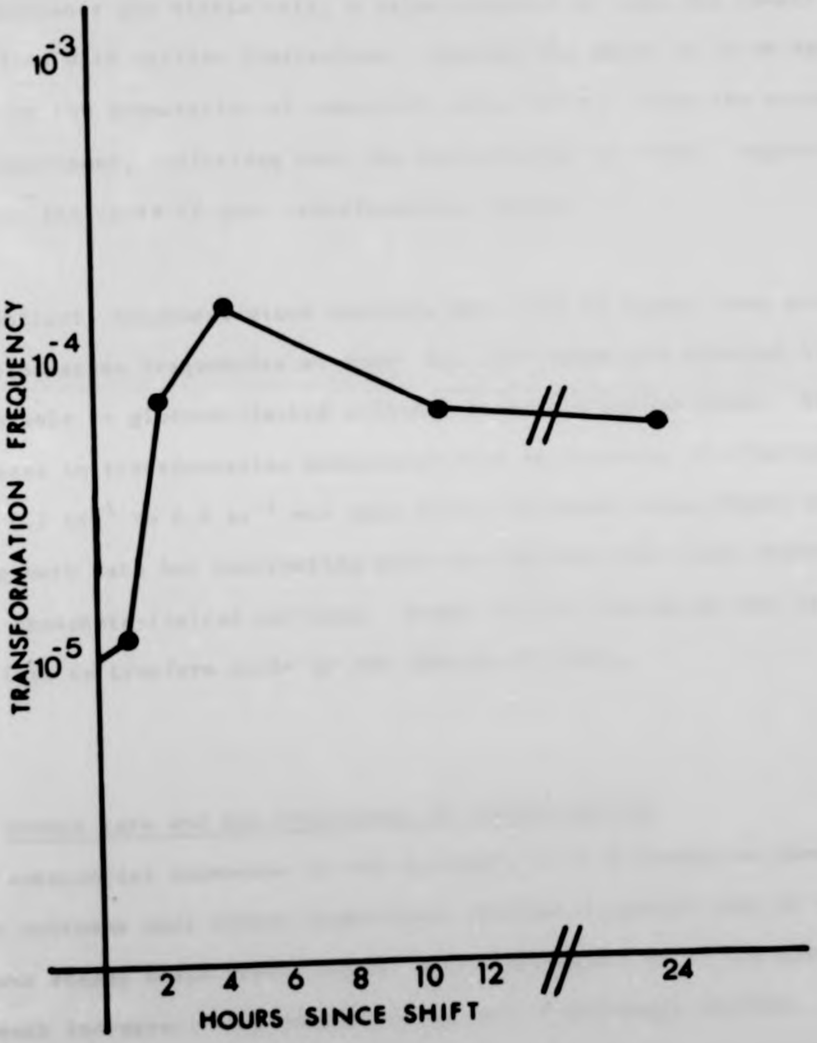
ix) $Mg^{2+}$ and sulphur limited cultures and the efficiency of transformation

In addition to the limitations of phosphorus, glucose and nitrogen used to control the growth of E. coli W5445 the effect of growth rate was examined on the transformability of cultures limited for either magnesium (as $Mg^{2+}$ or sulphur (as $SO^4$).

At a variety of dilution rates ranging from 0.1 $hr^{-1}$ to 0.8 $hr^{-1}$ samples were taken, stored as described and transformed with saturating amounts

Figure 29. Transformation frequencies of E.coli #5445 after a shift-up in growth rate and stabilisation of the new steady state.

A glucose limited culture of E.coli #5445 maintained at a dilution rate of 0·25 $hrs^{-1}$ was shifted to 0·55 $hrs^{-1}$ in a similar manner to that described for figure 26.
The changes in transformation frequency were followed throughout the shift-up period and into the new steady state level.



TRANSFORMATION FREQUENCY
$10^{-3}$
$10^{-4}$
$10^{-5}$
2 4 6 8 10 12 24
HOURS SINCE SHIFT

of plasmid DNA. The results obtained for each culture are shown in Figure 30. With cultures growing under magnesium limitation the levels of transformation were depressed over the whole range of dilution rates tested. The frequency of transformation did not rise above $10^{-5}$ transformants per viable cell, a value observed at only the lowest dilutions with earlier limitations. Washing the cells in 50 mM $MgCl_2$ prior to the preparation of competent cells did not alter the outcome of the experiment, indicating that the availability of 'free' magnesium was not the cause of poor transformation levels.

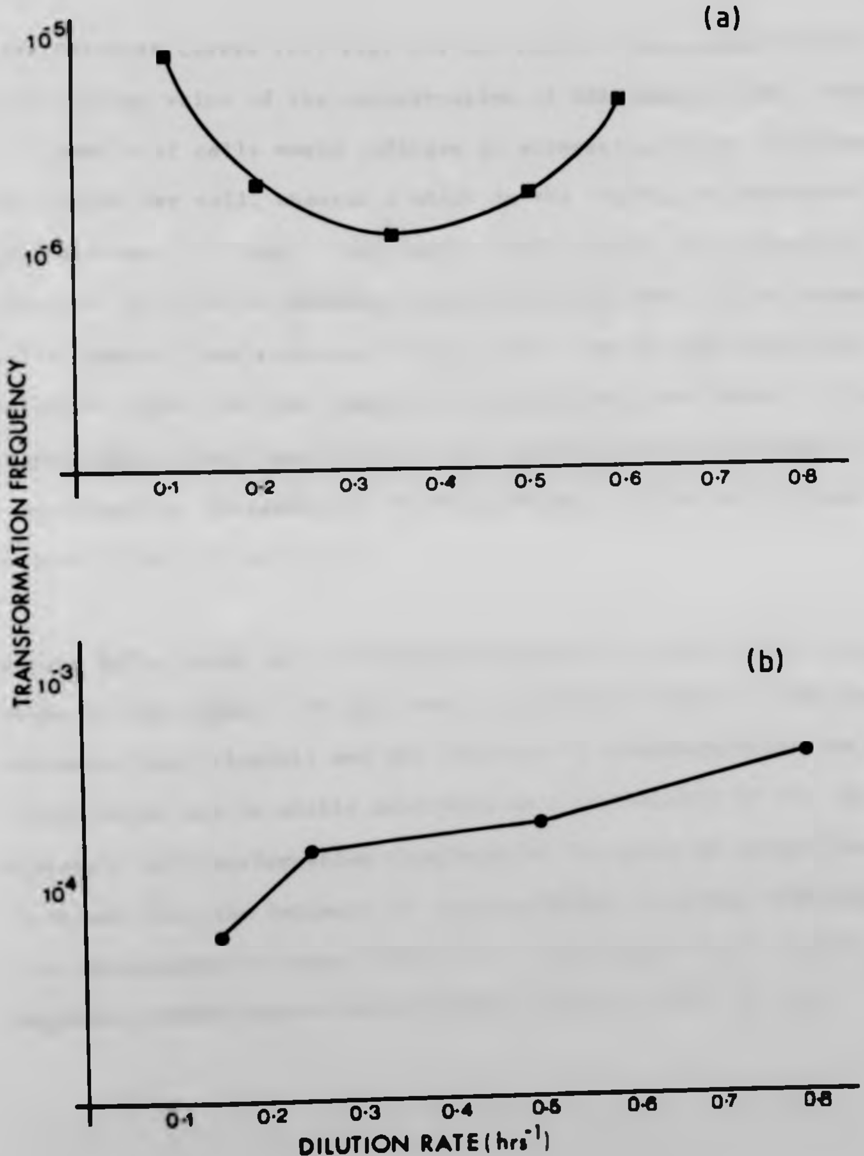
By contrast, sulphur-limited cultures gave rise to higher than normal transformation frequencies at lower dilution rates and attained levels comparable to glucose-limited cultures at high dilution rates. The increase in transformation associated with an increase of dilution rate from 0.1 $hr^{-1}$ to 0.8 $hr^{-1}$ was only 5-10 fold paralleling almost exactly the growth rate but contrasting with the 100-fold increases observed with phosphate-limited cultures. Under neither limitation was it possible to tranform cells in the absence of $CaCl_2$.

x) Growth rate and the efficiency of transformation

The substantial increases in the frequency of transformation observed when cultures were either transiently shifted in growth rate or moved to higher steady state growth rates, raised questions as to the mechanism of such increases. Two possibilities were immediately obvious, (1) that only a small proportion of each population was able to be transformed and that the relative proportion of that population increased with growth rate, or (2) that all cells have an equal probability of being

<u>Figure 30.</u> Transformation frequencies at differing dilution rates for magnesium limited and sulphur limited *E.coli* W5445.

Transformation frequencies at each growth rate were obtained as described for figure 25. The cultures were either magnesium limited ( a ) or sulphur limited ( b ).


(a)
10⁻⁵
10⁻⁶
TRANSFORMATION FREQUENCY
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
(b)
10⁻³
10⁻⁴
0·1
0·2
0·3
0·4
0·5
0·6
0·7
0·8
DILUTION RATE (hrs⁻¹)

transformed and that the probability increases with growth rate, i.e. each cell becomes easier to transform.

Dose response curves (cf. Fig. 23) can resolve these possibilities: a shift in the value of the concentration of DNA required for saturation of a sample of cells would indicate an alteration in the efficiency of DNA uptake per cell, whereas a shift in the plateau at saturation would indicate more (or less) transformable cells within the poplation. Dose response curves were therefore constructed for each of four samples of cells sampled from a glucose limited chemostat at four different dilution rates, and the results of this analysis are shown in Figure 31. Figure 31(a) shows that the four cell samples gave an increase in transformation frequency of 50-fold during a change of dilution rate from 0.16 $hr^{-1}$ to 0.74 $hr^{-1}$.

Figure 31(b) shows the dose-response curves for each of the samples shown in fig. 31(a). In each case saturation occurred at the same DNA concentration (~2µg/ml) and the increase in transformability at higher growth rates can be wholly explained as a consequency of the raised plateau's of transformation frequency at the point of saturation. That is to say that the increase in transformation frequency with growth rate is a consequence of there being more transformable cells within the poplation rather than a more efficient uptake of DNA per cell.

<u>Figure 3I.</u> Dose response curves at different dilution rates.

Samples of <u>E.coli</u> W5445 taken at each of four different dilution rates from a glucose limited chemostat were stored as described for figure 25. Transformation frequencies shown in figure 3I (a) were obtained by using saturating levels of plasmid DNA as defined earlier. In addition, each sample was transformed with a range of concentrations of pBR322 DNA in order to generate dose response curves of the type shown and described in figure 23.

Dose response curves (fig.3I (b)) were constructed for samples taken at dilution rates of:

0·I6 $hrs^{-I}$ ---- (▲)
0·3 $hrs^{-I}$ ---- (●)
0·5 $hrs^{-I}$ ---- (▼)
and 0·74 $hrs^{-I}$ ---- (◆).

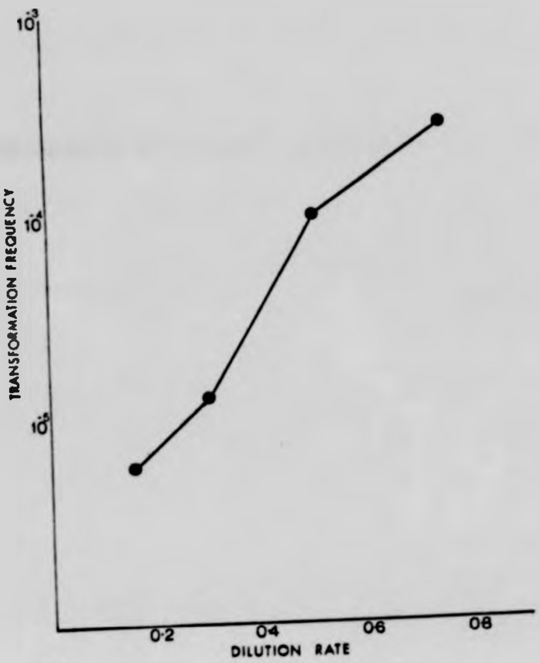
Figure 3I. Dose response curves at different dilution rates.

Samples of E.coli W5445 taken at each of four different dilution rates from a glucose limited chemostat were stored as described for figure 25. Transformation frequencies shown in figure 3I (a) were obtained by using saturating levels of plasmid DNA as defined earlier. In addition, each sample was transformed with a range of concentrations of pBR322 DNA in order to generate dose response curves of the type shown and described in figure 23.

Dose response curves (fig.3I (b)) were constructed for samples taken at dilution rates of:

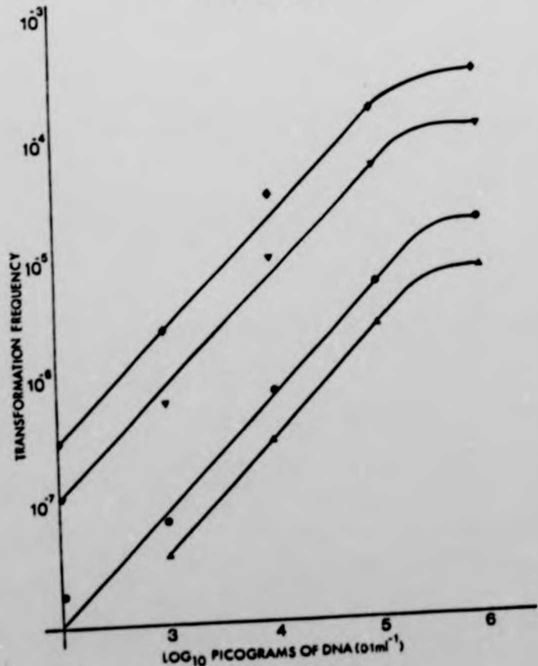
0·I6 $hrs^{-I}$ ---- (▲)
0·3 $hrs^{-I}$ ---- (●)
0·5 $hrs^{-I}$ ---- (▼)
and 0·74 $hrs^{-I}$ ---- (◆).

(a)



TRANSFORMATION FREQUENCY
DILUTION RATE


(b)



TRANSFORMATION FREQUENCY
LOG10 PICOGRAMS OF DNA

# VII Discussion of Results to Chapter VI

The experiments described in this thesis provide a novel examination of the effect of nutrient limitation and growth rate on the transformability of *E. coli* W5445. Under conditions of either glucose, nitrogen or phosphate limited growth a simple rule applied; the faster the population grew the more transformable it proved to be. In general the frequency of transformation could be raised 100-fold by a 10-fold increase in the growth rate. Phosphorus (as $PO_4^{=}$) limited cultures proved to be repeatedly more transformable than those growing under either glucose or nitrogen limitation, a result that suggests a repression of attainable transformation numbers when either of these two nutrients are limited. It is interesting to note that Humphreys *et al.* (1979) have noted a beneficial effect of glucose in the transformation buffer itself, these authors suggest that glucose provides a rapidly oxidizable substrate that aids the recovery of $CaCl_2$ treated cells after the transformation event. It is possible that poor survival of transformed cells limited for either glucose or nitrogen contributed towards the low levels of transformation when compared to cells grown under phosphate limitation.

Through the use of dose response curves constructed at a variety of growth rates, the increased number of transformants at higher growth rates was shown to be a consequence of there being more transformable cells in the population as opposed to a more efficient uptake of plasmid DNA per cell. Notani and Setlow (1974) have suggested that in some cases transformation can only occur at unique periods in the bacterial life cycle, for example at division. This cannot be the case for *E. coli* for although the number of dividing cells within a popuplation rises linearly with growth rate (Grover *et al.*, 1977) the observed rise in numbers of transformable cells is exponential. These results suggest that an additional factor, influenced by growth rate is directly involved is the ability of a cell to become transformed.

The average cell size of E. coli W5445 was shown to increase with growth rate, and the influence of cell size on transformability was examined. Cell size alone was found not to influence transformation frequencies by more than 3 or 4-fold; large (2 unit length) cells representing those about to divide were only 3-4 fold more transformable than 1 unit length cells (representing those that had just divided). This value of increase could be largely explained by assuming a limited number of DNA binding sites per cell surface, larger cells could therefore bind more DNA and their resultant chance of becoming transformed would be higher. The assertion (above) that transformation in E. coli does not occur at any one stage of the division cycle was further strengthened by the observation that no one period during the synchronous growth of E. coli W5445 was dramatically transformable; the stimulation observed prior to each wave of division substantiates the observation that larger cells are more easily transformed than smaller ones.

Taken together these results strongly suggest that cell size is not the second factor acting in synergy with growth rate to account for the increased numbers of transformable cells generated by increasing the growth rate.

Transformation of E. coli W5445 can be broadly split into two processes: uptake of DNA across the cell envelope layers and subsequent establishment of that molecule within the recipient cell. Either one of these steps could be subject to the phenotypic variation in transformation frequency observed by the alterations in nutrient limitation and growth rate employed in these experiments. Intracellular contents are known to change with the limitations used here (Tempest and Hunter, 1965; Neijssel and Tempest, 1975) and these could effect the establishment of any incoming molecule.

Several experimental points however indicate that the establishment of plasmids is unlikely to be the factor behind the observed changes in transformation frequency. Firstly, the dose response curves calculated for glucose limited cell populations showed that, no matter how poor the overall transformation frequency the slope of the curve was always unity; only one molecule was required to create a transformant. Secondly, the transformation protocol allowed a 90 minute 'expression' period in L-broth after the transformation event and prior to the selection of transformants. All cell samples thus recovered from the $CaCl_2$ treatment, a period during which the plasmid should begin to replicate and thus 'establish' itself, under identical conditions. Finally, although cotransformation experiments (Weston _et al_., 1979) have suggested interference between two jointly selected plasmids, such interference is not at the level of establishment; the interference levels observed with sets of compatible or incompatible plasmids were similar suggesting a competition for DNA binding sites during uptake rather than competition for cellular constituents after entry.

In the light of these date is is reasonable to propose that changes seen in transformation frequency during increases in dilution rate are not the result of an increase in the case of plasmid establishment, but rather the result of changes in cell wall structures that lead to altered access of DNA molecules to specific DNA binding sites present on the membrane. Based on this postulate, the changes in transformability observed with increasing growth rate could be explained as the result of a synergistic effect of growth rate and the permeability of the cell envelope. The results from the shift-up experiments described can also be explained on the basis of cell wall alterations. In shifted cultures, the levels of transformation achieved peaked just before the maximum growth rate or cell size at a point in the culture response that represented the most rapid point of change from the old growth rate to the new. At such a point cell wall synthesis will be at a maximum and presumably cell wall layers would be the minimum strength necessary for viability. In batch culture a similar point in the culture might occur as cells moved from the lag phase into the expontial phase, this is exactly the point when transformation frequencies are at their highest (Brown et al., 1979). Moreover, the finding that susceptibility to osmotic killing peaks at the same point strengthens the notion that cell walls are at their most fragile at this point in the growth phase (Brown et al., 1979).

What of the effects of magnesium and sulphate limitation?

Both the above conditions of nutrient limitation gave rise to atypical results when the affect of growth rate on transformability was examined. $Mg^{2+}$ ions have been found to stimulate transformation of E. coli with chromosomal DNA (Rejinders et al., 1978) and pretreatment with $Mg^{2+}$ ions can improve the $Ca^{2+}$ induced competence of S. typhimurium (Lederberg and Cohen, 1974) and P. aeruginosa (Mercer and Loutit, 1979). In addition, although the frequency is low, $Mg^{2+}$ ions can permit plasmid transformation of *E. coli* in the absence of $Ca^{2+}$ ions. In the light of these observations it is not too surprising that limitation for $Mg^{2+}$ results in a depression of transformation frequencies over the range of growth rates tested. However, the exact nature of the changes induced by magnesium limitation that cause the depression in transformation must await further biochemical analysis of cell populations growing under different nutrient limitations. It is possible that $Mg^{2+}$ ions act as an essential co-factor for enzymes obligatorily involved in DNA uptake or establishment or, that induction of $Mg^{2+}$ scavenging systems inhibits the uptake of DNA through the cell wall layers. Similar arguments can be invoked for the results under sulphate limitation. Indeed both $Mg^{2+}$ and $SO^4$ limitation are known to induce periplasmic proteins and so alter cell envelope layers (Lin , 1970).

It is interesting to note that limitation of a divalent cation ($Mg^{2+}$) led to a depression of transformation frequencies, whereas limitation of a divalent anion ($SO_4^=$) led to an increase in transformation frequencies. This result may suggest a role for membrane charge in the uptake of DNA, a possibility already noted by Humphreys *et al*. (1979).

During all the experiments described in this chapter, even under conditions that proved to be very favourable for the development of competence, no transformants were obtained in the absence of $Ca^{2+}$ treatment. It remains possible that under special growth conditions *E. coli* W5445 would exhibit natural competence, that is, that transformation could occur directly with cells removed from the chemostat, but consideration of the variety of parameters examined in although the frequency is low, $Mg^{2+}$ ions can permit plasmid transformation of *E. coli* in the absence of $Ca^{2+}$ ions. In the light of these observations it is not too surprising that limitation for $Mg^{2+}$ results in a depression of transformation frequencies over the range of growth rates tested. However, the exact nature of the changes induced by magnesium limitation that cause the depression in transformation must await further biochemical analysis of cell populations growing under different nutrient limitations. It is possible that $Mg^{2+}$ ions act as an essential co-factor for enzymes obligatorily involved in DNA uptake or establishment or, that induction of $Mg^{2+}$ scavenging systems inhibits the uptake of DNA through the cell wall layers. Similar arguments can be invoked for the results under sulphate limitation. Indeed both $Mg^{2+}$ and $SO^4$ limitation are known to induce periplasmic proteins and so alter cell envelope layers (Lin , 1970).

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During all the experiments described in this chapter, even under conditions that proved to be very favourable for the development of competence, no transformants were obtained in the absence of $Ca^{2+}$ treatment. It remains possible that under special growth conditions *E. coli* W5445 would exhibit natural competence, that is, that transformation could occur directly with cells removed from the chemostat, but consideration of the variety of parameters examined in these experiments make this appear unlikely. Continuous culture has also been used to study the transformation of Bacillus subtilis, an organism that does exhibit natural competence. In such experiments (see Lopez et al., 1975) B. subtilis became most competent at a 'key' dilution rate of 0.277 $hr^{-1}$, a value that optimized the number of transformable cells in the population and their length of stay in the chemostat.

The absence of any such 'key' dilution rate for the establishment of competent E. coli coupled with the inability of E. coli to be transformed in the absence of $CaCl_2$ suggests that the transformation process, or at least the early stages of it, can be more easily explained biophysically than biologically. This suggestion is consistent with those presented earlier relating levels of transformation to the permeability of the bacterial envelope. A possible scenario for the transformation process might then be as follows:

1) Cell populations are resuspended in $CaCl_2$ which is obligatory for high levels of competence. $Ca^{2+}$ ions presumably alter the permeability of the other cell layers although the observations made with $Mg^{2+}$ and $SO_4^{=}$ limited cultures may indicate the role of this ion in the neutralization of membrane charge.

2) Exogenously added DNA binds to a limited number of DNA binding sites on the cell surface. Although $Ca^{2+}$ ions are necessary for unveiling such binding sites, their numbers are influenced by the nutrient limitation used to limit growth and access to them may also be

limited by the speed at which cell wall material is being laid down.

3) Binding of DNA to these binding sites occurs in the cold when such sites (presumably proteins) are held immobile in a rigid membrane. At the heat shock, the membrane becomes fluid once more and those receptors that have bound DNA turn inwards to render the DNA resistant to the action of exogenous DNAase.

4) DNA is released into the periplasm or directly into cytosol, thereafter its survival is dependent on its ease of replication and resistance to degradation.

The above scenario could be considered as simply the biological consequence of essentially physical interactions, even the envisaged DNA binding to membrane embedded proteins may be opportunistic for the numbers of such proteins available for binding is evidently dependent on the growth rate and nutrient limitation.

At first glance such a scenario may appear too imprecise to warrant merit but the abundance of other forms of genetic exchange amongst strains of E. coli such as transduction and conjugation begs the question: Is transformation of E. coli a biologically evolved means of genetic exchange?

The results presented in this work suggest it is not, but continued use of continuous culture techiques to vary stable levels of competence will substantiate or contradict these findings.

PUBLISHED WORK

Molec gen Genet 180, 579-584 (1980)

MGG
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# Maintenance of Some ColE1-type Plasmids in Chemostat Culture

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**Summary.** When cells carrying the plasmids RP1, pDS4101 (a ColK derivative) or pDS1109 (a ColE1 derivative) were maintained in chemostat culture in the absence of antibiotic selection, plasmid-free segregants were not detected after 120 generations of nutrient-limited growth. By contrast, plasmid-free segregants of pMB9- and pBR322-containing cells arose after approximately 30 generations, irrespective of the host genetic background. However, even though pDS1109 was maintained its copy-number fell five-fold during 80 generations of limited growth. It is suggested that loss of pBR322 occurs following a similar copy-number decrease which results in defective segregation of the plasmid to daughter host cells. This defective segregation was not complemented in trans by either RP1 or pDS4101.

Molecular and General Genetics 180 : 579 - 584 (1980)

JOURNAL OF BACTERIOLOGY, June 1981, p. 841-846
0021-9193/81/060841-06$02.00/0 Vol. 146, No. 3

# Effect of Growth Rate and Nutrient Limitation on the Transformability of *Escherichia coli* with Plasmid Deoxyribonucleic Acid

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Received 24 October 1980/Accepted 11 March 1981

The observed transformation frequency by plasmid deoxyribonucleic acid of *Escherichia coli* grown in continuous culture was found to depend on both the steady-state growth rate and the type of nutrient used to limit growth. With carbon, nitrogen, or phosphorus limitation, the faster the growth rate, the higher the transformation frequency. The increase in transformation frequency associated with higher growth rates was shown to be due to more transformable cells in the population rather than an increased efficiency of deoxyribonucleic acid uptake. Growth rate had relatively little effect on the transformability of cells from sulfate- or $Mg^{2+}$-limited chemostats, indicating that some factor other than the growth rate must influence the frequency of transformation. Regardless of the nutrient limitation or the growth rate, no transformants were obtained in the absence of $CaCl_2$.

Journal of Bacteriology 146 : 841 - 846 (1981)

5

# Hereditary Instability of Recombinant DNA Molecules

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# APPENDICES

# APPENDIX 1

## Introduction

The introduction to bacterial plasmids presented in the main body of this thesis (pp1-10) moved rapidly from the discovery of plasmids to details of their segregational mechanisms. Consequently, some important aspects of plasmid biology, in particular plasmid replication and incompatibility, were dealt with only briefly.

This appendix provides additional information on these areas of plasmid biology intended to supplement that already presented in the main introduction.

## Incompatibility and Replication control.

It has already been stated (pp4-5) that the phenotypic observation of plasmid incompatibility is the result of the inability of two differentially marked but related replicons to co-exist within the same cell. That is, one or other of a pair of incompatible plasmids is prevented from replicating such that its copy number is insufficient to ensure segregation into both daughter cells. Incompatibility is thus intricately linked with the mechanisims of plasmid replication (i.e. copy number) control.

How could such inhibition of replication come about?

Two general models have been proposed to explain plasmid incompatibilty. The first is a derivative of the Replicon Hypothesis of Jacob et al.(1963) which states that occupation of a unique cellular site by a resident plasmid precludes binding to that site by a competing plasmid introduced into the same cell. As replication can only proceed when a plasmid molecule is bound to its replication site, the competing plasmid is lost during subsequent cell proliferation. Plasmids of the same incompatibilty (Inc.) group are thus defined as plasmids that are sufficiently similar to bind to the same cellular replication site.

An alternate hypothesis proposed to account for incompatibilty is the Inhibitor Dilution Model of Pritchard et al. (1969 and Pritchard, 1978) in which the inhibition of plasmid replication is mediated by a trans-acting repressor normally encoded by the plasmid itself. Inc. groupings in this case are of plasmids that share susceptibilty to the same replication repressor.

Some experimental data in favour of an "occupied site" model of incompatibilty (in particular amongst ColE1 type plasmids) has been published (Bedbrook et al., 1979) but these findings, which are at variance with others (e.g. Warren and Sherratt, 1978), have since been reinterprated by other workers (see Hashimoto-Gotoh and Timmis, 1981) to favour a model based on replication repressors. In addition, the Inhibitor Dilution theory predicts some features of plasmid replication that have been directly tested by several groups. For example, a model that predicts a constitutively synthesised repressor (Pritchard, 1978) suggests that the concentration of repressor will depend on copy number. It follows that alteration of plasmid copy number (e.g. by the selection of mutants) should lead to altered levels of incompatibility. This has indeed been shown to be the case for many plasmid systems studied (Uhlin and Nordstrom, 1975; Cabello et al., 1976; Pritchard, 1978; Hashimoto-Gotoh and Inselberg, 1979; Molin and Nordstrom, 1980). Further data supporting the Inhibitor Dilution Model concerns the trans-acting nature of the repressor. Using composite replicons based on two compatible component plasmids it has been shown (Timmis et al., 1974; Cabello et al., 1976; Meacock and Cohen, 1979) that each component of the chimera is able to express incompatibility irrespective of which component is undergoing replication. These results cannot be explained by an "occupied site" model which predicts that incompatibility should only be expressed by the member of the composite bound to a cellular replication site.

Although the Inhibitor Dilution Model explains adequately the repression of replication of one or other of an incompatible pair of plasmids, it does not fully explain (at least not in its simplest form) why one member of the pair is preferentially lost during subsequent growth. To account for this phenomenon, an extension to the model must be described.

A plasmid normally maintained at X copies per cell must undergo X replication events during each cell cycle. There are three possible mechanisms by which this can come about. First, a "master" copy of the plasmid replicates X times continually shedding "slave" plasmid copies into the cytoplasm. This is an extension of the "occupied site" model considered earlier. Second, every plasmid replicates once or, third, there is a random choice of template from the cellular pool at each new round of replication. This latter possibility (the Random Replication Model - Rownd, 1969) is the only model that easily fits the density - shift experiments described for a number of plasmids (Rownd, 1969; Bazaral and Helinski, 1970; Gustafsson and Nordstrom, 1975; Gustafsson et al., 1978).

Coupled with the Inhibitor Dilution Model, the random choice of template at each round of replication explains the preferential loss of one or other of a pair of incompatible plasmids during cell growth. In particular, it explains why (in general) resident plasmids are rarely replaced with an equivalent incompatible alternative (Hashimoto-Gotoh and Timmis, 1981): the resident, already present at high copy number is much more likely to be chosen for any rounds of replication allowed by dilution of the replication repressor in the growing cell. The single copy challenging replicon is thus continually diluted out of a growing population.

Inhibitor dilution and random template choice thus lead to a satisfactory explaination of incompatibility for many plasmid systems (Timmis, 1979; Timmis et al.,1981). Some relatively weak contibutions to the degree of incompatibity observed will be discussed later.

Identification of replication repressors.

Untill recently, evidence (see above) cited in support of the Inhibitor Dilution Model was primarily genetic; mutants could be isolated that had properties characteristic of alterations in the replication repressor itself or its target. Of late however evidence has been obtained for some plasmid systems that identifies both the agent of repression itself and, to a lesser extent, its mode of action (for a current review see Scott, 1984). This is particularly true for ColE1 type plasmids and those of the Inc.FII incompatibility group.

Initiation of replication in the colicin E1 plasmid (and its close relatives) occurs when a preprimer RNA molecule (RNA-II), which is transcribed from upstream of the replication origin, is pocessed by RNase H to provide a free 3' hydroxyl residue for the addition of deoxyribonucleotides by DNA polymerase I (Itoh and Tomizawa, 1980). The number of successful initiation events (and so rounds of replication) is thus dependant on the ease with which a stable preprimerRNA/DNA hybrid can form to provide a substrate for RNase H.

Determination of the DNA sequence of copy number/inc mutants of ColE1 (e.g. Muesing et al.,1980) has revealed changes in an area of ColE1 DNA known to encode a small RNA molecule (RNA-I) (Tomizawa et al., 1981) transcribed from the opposite strand of the DNA encoding RNA-II. The possibility that, because of this complementarity, RNA-I and RNA-II hybridize thus preventing successful RNase H processing at the origin (and so replication) has been essentially proven by a wealth of experiments using an in vitro ColE1 replication system (Tomizawa and Itoh, 1981; Tomizawa et al., 1981; Tomizawa and Itoh, 1982) and an extensive genetic and physical analysis of a large number of cop/inc mutants of ColE1 (Lacatena and Cesareni, 1981; Lacatena and Cesareni, 1983).

RNA-I has thus been identified as both a repressor of ColE1 replication and also the mediator of plasmid incompatibility.

A second level of ColE1 replication control is effected by the product of the rop gene (Cesareni et al., 1982) but this affect is independant of incompatibility (Twigg and Sherratt, 1980) and is probably either a direct repression of the synthesis of RNA-II or an aid to the interaction between RNA-I and RNA-II.

Control of ColE1 replication is currently the most well understood (Scott, 1984) but analogous regulation circuits appear to exist for the well studied Inc.FII group of plasmids. In this case, replication is dependant (unlike ColE1) on continued protein synthesis and in particular on the expression of the plasmid encoded Rep A1 protein. The translation of this protein depends on the absence of a small RNA molecule (RNA-E) that can bind to the Rep A1 message so preventing efficient translation. RNA-E (the product of the cop A locus) is thus the functional equivalent of the ColE1 RNA-I molecule and is the agent primarily responsible for Inc.FII plasmid copy number control and incompatibility (Timmis et al., 1981; Scott, 1984).

The understanding of replication control in other plasmid systems, whilst less well advanced, is also consistant with a negative control of replication mediated by the repression of an agent essential for replication (Scott, 1984). Without doubt, continued research into a variety of plasmid replication control systems will lead to a level of understanding for all plasmids as great as that now held for ColE1.

## Partitioning.

A good deal of discussion on the possible mechanisms of plasmid partitioning has been presented earlier (pp 6-10 and pp 91-96) but, in the main this comment centred on a comparison of the partition results of this work with that suggested for other plasmid systems. Recently, more information has become available on the nature of the partition systems of three different plasmids and, in each of these systems (P1 - Austin et al., 1981; CloDF13 - Hakkaart et al., 1982; ColE1 - Summers and Sherratt, 1984) a role in partitioning has been suggested for the site specific resolution of multimeric plasmid forms.

The recA protein of E.coli is normally involved in the generation/resolution of plasmid multimers and, in the absence of this protein (in recA strains) some plasmids are maintained in a "fixed" multimeric form (Bedbrook and Ausubel, 1978). Plasmids such as CloDF13 and ColE1 however are rarely found as multimers even after transformation of a recA strain (Summers and Sherratt, 1984) unless they have lost a specific function (designated cer for ColE1 and parB for CloDF13) that promotes the recA and recF independent conversion of plasmid multimers to monomers. As the Inhibitor Dilution Model (see earlier) of replication control counts origins irrespective of their molecular conformation, multimers exist in the cell at a lower copy number than the monomeric form. It follows that as the copy number falls (with increasing multimerisation) the chance of a plasmid free cell arising increases dramatically (see Table 6). Resolution of multimers by the action of cer or similar agents therefore increases plasmid stability and gives a par$^{+}$ phenotype.

A similar situation has been found in the large low copy number plasmid P1 (Austin et al., 1981) in which the absence of a dimer resolution site (loxP) leads to low level instability (Sternberg and Austin, 1981; Austin et al., 1981).

Whilst the resolution of multimers is therefore proven to be linked with efficient partitioning, it is likely not to be the only factor involved (not at least for all plasmids). Further analysis of the inheritance of the P1 plasmid has revealed a more prominent locus (par), the absence of which leads to only random partitioning (Austin and Abeles, 1983).
The P1 par determinant lies close to but seperate from the replication determinant and encodes a protein of 44,000 Daltons molecular weight whose action at a site (incB) is necassary for efficient partition to occur. As the genotypic prefix suggests, incB is an incompatibility determinant that is genetically distinct from the replication repressor/target interaction

described earlier. The incompatibility of incB likely results from competition for a unique cellular partition site, the exclusion from which leads to inefficient partitioning and plasmid loss. In support of this concept a weak form of incompatibility has been shown to occur between two otherwise compatible plasmids that contain common par elements (Nordstrom et al., 1981).

The criteria for efficient partitioning therefore appear to be (at least) twofold. First, a site specific recA independent resolution system ensures that there are enough individual molecules on which a partition system can act. Second, after replication and/or resolution, a specific interaction between a par genetic locus and a cellular site occurs such that at least two randomly chosen plasmid molecules are bound and accurately partitioned, one to each daughter cell.

It may well be that for high copy number plasmids such as ColE1, resolution of multimers is sufficient to ensure partitioning by the random assortment of plasmids at division. However, the results (above) for the P1 plasmid coupled with those reported for the F factor (Ogura and Hiraga, 1983) indicate that, at least for some plasmids, both resolution and subsequent membrane binding are necessary for efficient partitioning.

Other factors.

This appendix has detailed recent advances in the understanding of both plasmid replication control and partitioning. It is worthy of note however that other factors, particularly the transcriptional activity of a plasmid can interfere with both of these functions. Stueber and Bujard (1982) have done an extensive analysis of the effect of strong transciption signals (e.g. unregulated bacteriophage promoters) on plasmid copy number and stability. They conclude that transcription mediated instability is caused by both an overproduction of plasmid encoded proteins, some of which (e.g. the rop protein -see above) maybe inhibitory for plasmid replication or cell growth, and a direct interference (by transcripts opposing the growing replication fork) in the formation of message (e.g.RNA-II) necassary for replication. Inactivation of some reading frames and, more importantly, the integration of powerful transcription terminators downstream of active promoters leads to both an increase in plasmid copy number and stability.

Interference by plasmid specified transcripts has also been forwarded as the reason why the pSC101 par element (Meacock and Cohen, 1980) fails to completely stabilise derivatives of either pBR322 or pACYC184 that carry the E.coli trp operon (Skogman et al., 1983). As each of these vectors has been stabilised by pSC101 par in the absence of any insert (see chapter IV; Meacock and Cohen, 1980; Primrose et al., 1984), transcripts from within the trp operon must confere the unstable phenotype. Whether or not the instability described by these authors is due to low copy number (for example after multimerisation) or to a direct effect of transcription readthrough of the par locus has yet to be determined.

# APPENDIX II

A detailed protocol for the induction of competence in E.coli W5445, as used throughout the transformation work described in this thesis is given below.

Cell samples from either batch cultures (harvested at an absorbance of 0.3 (600nm)),from frozen stocks or taken directly from the chemostat were chilled and pelleted at 5000rpm for 15 minutes at 4°C.

The cell pellet was resuspended in a solution of ice cold 30mM $CaCl_2$ to a volume one half that of the original culture and left on ice for 20 minutes.

The chilled cell suspension was then repelleted (as above) and finally resuspended in a solution of ice cold 75mM $CaCl_2$ to a volume one twentieth that of the original culture. The now competent cell preparation was kept on ice until used.

The proceedure followed for the transformation itself has been described earlier (pp34-35).

All the transformation work descibed in this thesis used a competence inducing protocol (above) that used only $CaCl_2$. Recently, a more elaborate mixture of compounds (including $CaCl_2$) has been shown to increase the transformation frequency of several E.coli strains (Hanahan, 1983). Interestingly, many of the conclusions reached in this thesis work, particularly the relationship between growth rate and transformation frequency are supported by the experiments of Hanahan (1983) using the improved competence proceedure.

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