Factors Involved In DNA Replication In *Escherichia coli*: The *dnaA*, *groE* and *pcn* Gene Products

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To My Parents

Abstract

An investigation into the mechanism by which over-expression of the *groE* genes is able to suppress temperature sensitive mutations in the *dnaA* gene in *Escherichia coli* has been conducted. The available data would tend to suggest that suppression is due to a specific interaction between the *groE* and *dnaAts* gene products.

Suppression is allele specific; mutations which map to the centre of the gene are suppressed; those at the ends are not. The specificity of allele suppression is identical to that exhibited by certain mutations in the *E.coli rpoB* gene. Suppression requires the presence of the mutant DnaA protein; *dnaAamb* mutants are not suppressed. Using fusions of P*dnaA-lacZ* it has been shown that excess GroE does not act directly to increase transcription of the *dnaA* gene.

A β-galactosidase-DnaA fusion protein has been constructed and used to raise DnaA antiserum. Induction of this fusion protein at high temperature leads to cell death. It is unclear whether the fusion protein possesses DnaA activity. The DnaA antiserum has been used to show that there are less than 1000 molecules of DnaA protein per cell. While attempting to clone the *dnaA* gene, a large number of 'partial' high-copy suppressors of *dnaAts* were isolated (so called because they result in better growth at higher temperature, while not significantly increasing the maximum temperature at which growth is possible). The *rpoBC* genes appear to fall into this catagory.

Over-expression of *groE* in conjunction with certain *dnaAts* alleles causes cold sensitivity. This is allele specific; all cold sensitive alleles can also be suppressed by *groE*. The pattern of allele specificity is closely matched by the *'oriC'* sensitivity of *dnaAts* alleles (that is, reduced growth when transformed with an *oriC* plasmid. Over-expression of *groE* results in numerous other side effects to the cell. These include; a reduced resistance to the antibiotics nalidixic acid and ampicillin; increased resistance to streptomycin; and abnormal sensitivity to heat shock. An investigation into these effects has been conducted.

A plasmid containing *groES* and a truncated *groEL* has been constructed. This plasmid is maintained at a low copy number, yet is able to suppress *dnaAts* mutations with no apparent over-production of GroE. The deletion in the GroEL protein has removed a glycine rich region located at the carboxyl terminus. The significance of this deletion regarding the ability of GroE to suppress *dnaAts* is discussed, in the

light of the appearance of such a glycine rich sequence in a variety of structural proteins (keratins, viral coat proteins and heat shock proteins).

A novel mutation which results in a reduced copy number for pBR-type plasmids and their derivatives has been isolated and characterised. The mutation, called *pcn* lowers the copy number of pBR-type plasmids to 25% of wild type levels. It has been mapped to 3.6 minutes on the *E.coli* genetic map; on the *E.coli* physical map it has been located between *panD* and *fhu*. The gene encodes a protein with an apparent molecular wieght of 48kd, and has been shown to be allelic to two other independently isolated (but incompletely characterised) plasmid copy number mutations. Replicons which are affected by *pcn* include pMB1, ColD, p15A, pSC101 and R1. F and *oriC* replication appears unaffected, as is the lytic DNA replication of a variety of bacteriophages.

An investigation into the technique of quantitative hybridisation as a means of measuring plasmid copy number has been undertaken. Various parameters have been examined (method of cell lysis; linearity of response; effect of altering the region of homology), and a description of a useful experimental technique which gives results as plasmid concentration, plasmids per cell, or plasmids per chromosomal marker (e.g. *oriC*) is presented.

Declaration

I declare that the work contained in this thesis is my own unless otherwise stated.

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Contents

Abbreviations

ADP	adenosine 5'-diphosphate
AMP	adenosine 5'-monophosphate
amp	ampicillin
AMPS	ammonium persulphate
ATP	adenosine 5'-triphosphate
bp	base pairs
BSA	bovine serum albumin
cAMP	cyclic adenosine 5'-monophosphate
chl	chloramphenicol
CsCl	caesium chloride
cpm	counts per minute
DNA	deoxyribonucleic acid
dsDNA	double-stranded DNA
DTT	dithiothreitol
E.	*Escherichia*
EDTA	diaminoethanetetraacetic acid
EtBr	ethidium bromide
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid
IPTG	isopropyl-β-D-thiogalactoside
kan	kanamycin
kb	kilobase
kd	kilodaltons
mRNA	messenger RNA
OD_w	optical density $_{\text{wavelength}}$
PAGE	polyacrylamide gel electrophoresis
ppGpp	guanosine tetraphosphate
rif	rifampicin
RNA	ribonucleic acid
rpm	revolutions per minute
SDS	sodium dodecyl sulphate
ssDNA	single-stranded DNA
str	streptomycin
tet	tetracycline
TEMED	N,N,N',N'-tetramethylethylenediamine
Tris	tris(hydroxymethyl)aminomethane

v:v	volume by volume
w:v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactoside
x^R	resistance to antibiotic X

Preface

As the title of this thesis indicates, the subjects of study are the *dnaA, groESL* and *pcnB* gene products of *Escherichia coli*. For the most part the thesis can be regarded as being comprised of two quite separate research topics: that of an investigation into the mechanism by which over-expression of the *groE* genes is able to suppress *dnaA* temperature sensitive mutations, and secondly, the isolation and identification of a chromosomal locus (*pcnB*) which results in a lowering of plasmid copy number. Because of this division, it has not proved possible to present the thesis as a straightforward study of a single subject matter.

The research project originally began as an investigation into the mechanism by which over-expression of the *groE* genes is able to suppress *dnaAts* mutations. The subsequent isolation of the *pcnB* mutant was a direct consequence of the cold sensitive phenotype of the *dnaA46ts* allele in conjunction with the over-expressing *groE* plasmid pND5 (discussed in chapter 4); other than this, the topics share little common ground. As a result, the *pcnB* mutation is considered in more detail as a self-contained unit in chapter 7, with the necessary introduction, results, and discussion contained within. In an analogous manner, the chapter on quantitative hybridisation (chapter 8) concerns a quite separate subject matter from the rest of the thesis, and is essentially concerned with the methodology of the technique. Like chapter 7 it should be considered self-contained, and is not covered by the introductory or discussion chapters of this thesis (chapters 1 and 9).

This frees the rest of the thesis for a discussion of the interactions between the *groE* and *dnaA* gene products, and the subject is presented as follows:-

- Chapter 1 is an introduction to the project, and covers DNA replication (with a particular emphasis on the *dnaA* gene product and its role in the initiation process), together with an assessment of the current state of knowledge on the *groE* gene products.

- Chapter 2 details the materials and methods used for the entire investigation (that is the *groE-dnaA* research topic, the *pcnB* research topic, and the quantitative hybridisation experiments).

- Chapter 3 concerns the subcloning of the *groE* genes and an investigation into homologies between GroEL and other proteins (including an assessment of the possible significance of these homologies).

- Chapter 4 is mainly concerned with a genetic analysis of the mechanism of suppression, and includes a section on the cold-sensitivity phenotype (which was the basis for isolating the

pcnB mutant described in chapter 7).

- Chapter 5 covers the cloning of the *dnaA* gene on a multicopy plasmid vector, together with the construction of a *lacZ-dnaA* gene fusion. This was subsequently used to produce purified fusion protein which was used to raise DnaA antiserum. A preliminary description of a variety of partial *dnaAts* suppressors obtained during these experiments is included also.

- Chapter 6 concerns an investigation into the multiple phenotypic effects resulting from over-expression of the *groE* genes, and attempts to discuss their relevance towards understanding the process of suppression.

- Chapter 9 is summary and discussion of the results of chapters 3-6.

Due to the complexity of the subject matter, together with the wide range of topics covered, each of the above results chapters includes a discussion as an integral part of it, rather than attempting to discuss all the experimental results as a single unit at the end. In effect then, chapter 9 contains summaries of the results and conclusions of chapters 3-6, and from this an assessment of the most likely mechanism by which suppression is occurring is made.

CHAPTER 1

Introduction: background to the *dnaA* and *groE* gene products and their role in the cell

1.1 An Overview Of DNA Replication In *E.coli*

1.1.1 Introduction

Replication of the *E.coli* chromosome proceeds bidirectionally from a fixed origin of replication, *oriC*, located at 84 minutes on the genetic map (Masters and Broda 1971, Bird *et al.* 1972, von Meyenburg *et al.* 1977, Bachmann 1983). Elongation of the daughter chromosomes then occurs, leading to termination at a site, *terC*, located diametrically opposite *oriC* (Bouche 1982). The period during which chromosome replication occurs is known as the C-time; the period between termination and cell division is the D-time (Helmstetter *et al.* 1968). With the exception of very slow growth rates, the C and D times are relatively constant; 40 minutes and 20 minutes respectively at 37°C (Helmstetter *et al.* 1968, Churchward and Bremer 1977). Thus for cells growing with a doubling time of less than 40 minutes, additional rounds of replication must begin prior to completion of the previous round, leading to nested replication forks and an increased origin to terminus marker ratio. Since the rate of chain elongation remains constant, overall control of DNA synthesis must lie with the initiation frequency. Although it has been shown (Donachie 1968) that cells initiate DNA replication when they reach a fixed volume or mass, known as the 'Initiation Mass', or a binary multiple thereof, the molecular mechanism by which achievement of this 'Initiation Mass' triggers DNA replication is far from clear.

Models based on the dilution of a periodically synthesised repressor molecule (Pritchard *et al.* 1969), or the accumulation of a constitutively expressed initiator molecule (Jacob *et al.* 1963, Helmstetter *et al.* 1968) have been suggested, and on current evidence are both tenable. They are of interest in this study, since regulation of initiation is one of the functions ascribed to the DnaA protein (Atlung *et al.* 1985b). Although the ability of the DnaA protein to act in a regulatory capacity has been clearly demonstrated by several groups (Atlung *et al.* 1985a; Braun *et al.* 1985; Lother *et al.* 1985, Kucherer *et al.* 1986, Wang and Kaguni 1987), its exact role in the regulation of initiation is less clear. However, despite a certain amount of confusion in the past concerning whether DnaA acted as a positive or negative element in specific aspects of the initiation process (Atlung *et al.* 1985b, Churchward *et al.* 1983, Rokeach and Zyskind 1986), the general consensus of opinion now seems to be graduating towards the protein having a positive action in controlling the overall level of initiation (Atlung *et al.* 1987, Xu and Bremer 1988). That is, DnaA protein serves to stimulate initiation, and thus might be considered a candidate for the 'Inititiator Molecule' of the model of Helmstetter *et al.* (1968). However, despite the acceptance of the DnaA

protein in general terms as a postively-acting initiator element, specific details of its effects and interactions are less clear; for example, the effect of over-expression of the *dnaA* gene on the frequency of initiation (Atlung *et al.* 1985a, 1987; Xu and Bremer 1988). Any attempt therefore, to conduct an appraisal of the role of the DnaA protein in the cell should be approached with a certain degree of caution.

1.1.2 Leading and lagging strand initiation

Synthesis of DNA is semi-conservative (Meselson and Stahl 1958), and proceeds through elongation of DNA chains complementary to the parental template strands. Since all known DNA polymerases can only synthesise DNA in a 5'-3' direction, synthesis of lagging strand DNA requires intermittent synthesis of an RNA primer, followed by extension of this primer by DNA polymerase III (for detailed reviews of the process of DNA replication see Kornberg 1980, 1982, and McMacken *et al.* 1987). Thus it becomes apparent that two different kinds of initiation event occur within the general context of DNA replication; that of the leading strand, which occurs at the origin and takes place only once per chromosome replicated, and that of the lagging strand, which occurs many times. Although much of the replication machinery and the biochemical events involved are highly similar, the details of the processes differ. Such details may give valuable insights into the mechanisms by which the unique initiation of leading strand synthesis and multiple initiations of lagging strand synthesis can take place.

- Initiation of leading strand synthesis is rifampicin sensitive, suggesting a requirement for RNA polymerase (Zyskind *et al.* 1977). In addition, a variety of *in vivo* (Bagdasarian *et al.* 1977, Tanaka *et al.* 1983, Atlung 1984) and *in vitro* (Ogawa *et al.* 1985, van der Ende *et al.* 1985, Sekimizu *et al.* 1988) studies have indicated a role for RNA polymerase in the initiation process. It is not clear whether the RNA polymerase fulfils the same role as that played by DNA primase in lagging strand synthesis (i.e. synthesis of an RNA primer), or whether it fulfils some other role (e.g. separating DNA strands; transcription of an 'initiator' gene).

- Leading strand initiation requires protein synthesis, and can be blocked by amino acid starvation or the addition of chloramphenicol (Lark *et al.* 1963, Maaloe and Hanawalt 1961), suggesting a possible requirement for an 'Initiator' protein.

- Leading strand initiation absolutely requires the participation of the DnaA protein, both *in vivo* and *in vitro*, with this protein being required at no other time during the replication cycle (Hirota *et al.* 1970; Fuller *et al.* 1984). This is in contrast to the products of other replication genes such as *dnaB* and *dnaC*. Although some mutant alleles of these genes exhibit the slow-stop phenotype characteristic of initiation mutants (Zyskind *et al.* 1977, Zyskind and Smith 1977),

elongation defective mutants can be found also (Wechsler 1978), indicating a role in both the initiation and elongation processes for their gene products. Thus the products of the *dnaB* and *dnaC* genes play a role in both initiation and elongation, although the existence of two classes of mutant would suggest a somewhat different role in each case.

1.1.3 Proteins involved in DNA replication; the replisome and associated proteins

Since DNA polymerase can only synthesise DNA in a 5'-3' direction, the opposing polarities of the two strands of the DNA helix means that replication must necessarily proceed in a different manner dependant upon which strand is being duplicated. On the basis of the high processivity of the DNA polymerase III holoenzyme, the strand complementary to the parental 3'-5' template is assumed to be synthesised continuously following a single priming event (Fay *et al.* 1981, Kornberg 1988). In contrast, lagging strand replication is synthesised in a retrograde 5'-3' direction in pieces of c.2000bp (Okazaki *et al.* 1968; Sugino *et al.* 1972) which are then rapidly ligated together. As a result of this, lagging strand replication requires intermittent synthesis of an RNA primer by DNA primase (the product of the *dnaG* gene; Rowen and Kornberg 1978), since DNA polymerase is unable to initiate strands *de novo*, but can only extend pre-existing ones (cf. Kornberg 1980; 1982). At present it is unclear whether RNA polymerase or DNA primase is responsible for laying down the RNA primer required for the (presumed) single priming step in leading strand initiation.

Kornberg (1982) has suggested that priming and elongation may be coupled through the assembly of a 'Replisome', containing the enzyme responsible for DNA replication, the DNA polymerase III holoenzyme (Gefter *et al.* 1971, Kornberg and Gefter 1971), and a primosome complex. The primosome complex is proposed to take part in the priming reactions which are an essential part of lagging strand synthesis, while the DNA polymerase III holoenzyme is involved in the actual DNA synthesis. Although evidence for the existence of a 'Primosome Complex' is largely circumstantial, being based on the fact that many of its components are known to co-purify or interact *in vitro* (Wickner and Hurwitz 1975; Arai and Kornberg 1979; McHenry and Kornberg 1977, Selick 1987, Maki *et al.* 1988, Kornberg 1988), the spacial and temporal closeness of the reactions occurring during DNA replication are a persuasive argument for the existence of some kind of co-ordinating structure.

1.1.3.1 DNA polymerase III holenzyme and other replication proteins

Once the initiation process has been completed and DNA replication is underway (to be discussed later), the advancement of the replication fork depends on the unwinding of the duplex DNA by the action of helicases, and the subsequent relieving of the resulting positive supercoiling by the action of topoisomerases. At present it is unclear whether the rep helicase (Yarranton *et al.* 1979), in concert with a primosomal complex is responsible for this unwinding (as proposed by Kornberg 1982, 1988), or whether the DnaB helicase (LeBowitz and McMacken 1986), either singly or in complex with DnaG primase is responsible (as proposed by McMacken *et al.* 1987). The recent finding (Lasken and Kornberg 1988) that primosomal protein n′ is a DNA helicase suggests that it too may have a role to play in the unwinding reaction. Following the unwinding of the duplex DNA, single-strand binding protein (SSB) binds to the DNA, protecting it from nuclease action and preventing reannealing (Sigal *et al.* 1972, Lohman and Overman 1985). An additional consequence of this unwinding is the accumulation of positive supercoiling in the unmelted duplex upstream of the replication fork. This is relieved by the swivelling action of DNA gyrase, one of the three *E.coli* topoisomerasess and the one believed to play the major role in the replication of its DNA (see Drlica 1984 for review). DNA gyrase is comprised of two polypeptides (gyrase A and gyrase B), in equimolar amounts as an A_2B_2 tetramer. It functions by making transient double stranded breaks in the DNA and introducing negative supercoils by passing one strand over the other in an as yet unclear process, the energy to do this coming from the hydrolysis of ATP (Sugino *et al.* 1978, Brown and Cozzarelli 1979).

Polymerisation is carried out by the DNA polymerase III holoenzyme, a large multiprotein complex consisting of (at the latest count) no less than 10 different subunit polypeptides (Maki *et al* 1988). Catalytic activity resides in the α subunit (132kd), the product of the *dnaE* gene (Spanos *et al.* 1981). Auxiliary subunits include β (37kd), ε (27kd), θ (10kd), τ (71kd), γ (52kd), δ (35kd), δ′ (33kd), χ (15kd) and ψ (12kd), and are primarily concerned with the processivity of the enzyme (β subunit), the 3′-5′ exonuclease activity (ε subunit), or are of unknown function (O'Donnell 1987, Maki and Kornberg 1988). Genes thought to encode subunits include *dnaN* (β), *dnaQ* (ε), and *dnaZX* (τ; γ). The genes encoding subunits θ, δ, δ′, χ and ψ are as yet unidentified (Maki *et al.* 1988). On the basis that the DNA polymerase III holoenzyme appears to be organised as an asymmetric dimer (Maki *et al.* 1988), Kornberg (1988) has suggested that this may endow each core polymerase with different properties, one suited to leading strand synthesis and the other lagging strand synthesis as part

of a dimeric complex at the replication fork in conjunction with the primosome (see below).

1.1.3.2 The ϕX174 primosome

The primosome complex was originally proposed following early *in vitro* DNA replication experiments on the conversion of single-stranded ϕX174 viral DNA into the duplex replicative form (Schekman *et al.* 1974, Wickner and Hurwitz 1974, Arai and Kornberg 1981a, 1981c, Arai *et al* 1981b). This was taken as a model for the molecular mechanisms involved in the discontinous synthesis of the lagging strand during replication of the *E.coli* chromosome (Kornberg 1980, 1982). While not necessarily being an accurate representation of the events taking place during leading strand initiation at *oriC*, it has nonetheless proved to be be highly illuminating regarding the general processes involved in lagging strand DNA synthesis.

The ϕX174 primosome is proposed to consist of a number of proteins, amongst them the products of the *dnaG* gene, the *dnaB* gene, the *dnaC* gene, proteins n, n', n" and i, together with the participation of numerous other ancillary proteins (DNA gyrase, SSB, HU, Helicases) (Kornberg 1980, 1982). The *dnaG* gene product (DNA primase) synthesises the RNA primers used in lagging strand synthesis (Rowen and Kornberg 1978), although it is not clear if it also fulfils this role in the primary initiation event at *oriC*. The *dnaB* gene product is known to exhibit helicase activity (Baker *et al.* 1986; LeBowitz and McMacken 1986), and it can form complexes with the DnaC protein (Kobori and Kornberg 1982a). Genetic evidence from studies of suppressor mutations has also indicated that DnaB and DnaC interact *in vivo* (Sclafani and Wechsler 1981, Maurer *et al.* 1984). The exact role of DnaB *in vivo* is unclear: in the early stages of initiation at *oriC* it has been proposed to form a 'preprepriming' complex with DnaC and DnaA, with the helicase activity of DnaB allowing localized unwinding of the duplex DNA prior to RNA priming (van der Ende *et al.* 1985, Baker *et al.* 1986, McMacken *et al.* 1987). As part of the ϕX174 primosome complex it is believed that either DnaB protein (McMacken *et al.* 1977) or protein n' (see below; Arai *et al.* 1981b) is responsible for the movement of the primosome complex along the DNA template by hydrolysis of rNTPs (LeBowitz and McMacken 1986).

The role of the DnaC protein is not clear; it appears to be involved in the assembly of the ϕX174 primosome, although a subsequent participation in the replication events has not been shown (Weiner *et al.* 1976, Arai *et al* 1981a, Kobori and Kornberg 1982b). Such an assembly role would agree with that postulated for DnaC in the initiation of replication at *oriC*, where DnaC is apparently to deliver the

DnaB protein to *oriC* by way of a DnaB–DnaC specific complex (Baker *et al.* 1986). Since both slow and fast stop mutants in DnaC exist (McMacken *et al.* 1987) it would seem likely that the role of this protein is somewhat different in the initiation and ongoing replication reactions. Protein i has recently been reported (Masai *et al.* 1986) as being the product of the *dnaT* gene, which is closely linked to *dnaC* and may form part of the same transcriptional unit (Lark and Lark 1978). The suggestion that protein i is in fact coded for by the *dnaT* gene seems eminently plausible, since the *dnaC2* mutation is partially suppressed by a secondary mutation in the *dnaT* gene (Lark and Lark 1978). This is not surprising if both protein i and *dnaC* are part of the primosome. In addition, *dnaTts* mutants display the slow-stop phenotype characteristic of initiation mutants (Lark and Lark 1978). The actual intracellular role of protein i is unclear; it is required for the assembly of the primosome complex, although it appears to have no intrinsic enzymic activity (Arai *et al.* 1981c, Weiner *et al.* 1976) and replication of *oriC* minichromosomes *in vitro* can be accomplished without this protein (Funnell *et al.* 1986). The role of protein n′ in initiation is far from clear. Replication of ϕX174 and ColE1 DNA is dependent upon the presence of protein n′ *in vitro*, with the protein apparently binding to the DNA at a specific sequence and thereby allowing subsequent primosome assembly (Arai *et al.* 1981a, 1981b, Nomura *et al.* 1982, Minden and Marians 1985). Two research groups have recently reported that protein n′ is in fact a DNA helicase (Lee and Marians 1987, Lasken and Kornberg 1988), and it has been suggested that in its role as part of the ϕX174 primosome it can be progressively translocated along ssDNA and destabilise and unwind any duplex regions which it may encounter. Regarding the role of protein n′ in *E.coli* chromosomal DNA replication, it had been assumed to be the major factor responsible for the mobility and helicase activity of the primosome, mainly due to its ability to hydrolyse dATP efficiently, in contrast to the low activity of the DnaB protein (Arai and Kornberg 1981a, 1981b; Arai *et al.* 1981a). However, these early studies were performed using single stranded phage or ColE1 plasmids as templates, for which protein n′ was found necessary for primosome assembly and subsequent displacement of SSB. Recent work by Baker *et al.* (1986) on minichromosomes containing *oriC* has demonstrated that these functions are in fact performed by the DnaB protein; indeed, they question the involvement of protein n′ in *E.coli* DNA replication, suggesting instead that its role might lie in phage or plasmid replication.

Like protein n′, proteins n and n″ have yet to be assigned a chromosomal locus. Similarly, their role in primosome structure and function is not known. They are required for its assembly, but it is not known what part they play, if any, in its subsequent functioning (Arai *et al.* 1981a).

1.1.3.3 Does the primosome have a role in *E.coli* DNA replication?

How accurate a picture is provided by the ϕX174 primosomal model for *E.coli* DNA replication? Phage ϕX174 DNA replication, with its associated primosomal multiprotein complex, has long served as a model for the processes involved in discontinous synthesis of the lagging strand during *E.coli* chromosome replication. The model has been supported by the finding that primosome assembly sites are present on certain *E.coli* plasmids which are known to utilse host-proteins in their DNA replication (Nomura and Ray 1980, Zipursky and Marians 1980, Imber *et al.* 1983), and it has been demonstrated that replication of pBR322 DNA *in vitro* is dependent upon the presence of primosomal proteins (Minden and Marians 1985).

However, despite this circumstantial evidence, there is no proof of an involvement of the ϕX174 primosome in *E.coli* DNA replication at all, and indeed, much of the available evidence would argue against it being a participant. No host mutations in the primosomal components n, n′ and n″ have yet been discovered, suggesting that these proteins may not participate in host cell DNA replication (hence there being no obvious phenotype available). Additionally, no primosome assembly sites are found near *oriC*, as would be expected if this complex were involved in the initial priming reaction (Kaguni and Kornberg 1984), although sites are found within a few kb (Stuitje *et al* 1984, van der Ende *et al.* 1983). Finally, recent biochemical studies have shown that *oriC* minichromosomes can be replicated in an *in vitro* system in the complete absence of the primosomal proteins n, n′ and n″, demonstrating that the intact ϕX174 primosome is not an absolute requirement for *E.coli* DNA replication *in vitro* (van der Ende *et al.* 1985, Funnell *et al.* 1986).

Of course the conditions in an *in vitro* replication system may not be a true representation of the events *in vivo*, where concentrations and cofactors may be significantly different. It is possible for example, that the ϕX174 primosome is the preferred system for priming the *E.coli* chromosome *in vivo*, although other mechanisms can be utilised if required. Alternatively, the primosome may indeed be an artifact as far as the study of components involved in *E.coli* DNA replication goes, being required for plasmid and phage DNA replication but not for that of the host cell. In this context, regarding the plasmid copy number mutant under study in chapter 7, it was originally considered that *pcnB* may in fact be the gene for one of the primosomal proteins n, n′ or n″. However, the molecular weight of PcnB appeared not to correspond with the measured sizes for any of these proteins (48kd for PcnB against 14kd, 76kd and 17kd for n, n′ and n″ respectively), making this hypothesis unlikely. A further possibility which should be considered is that the primosome is not

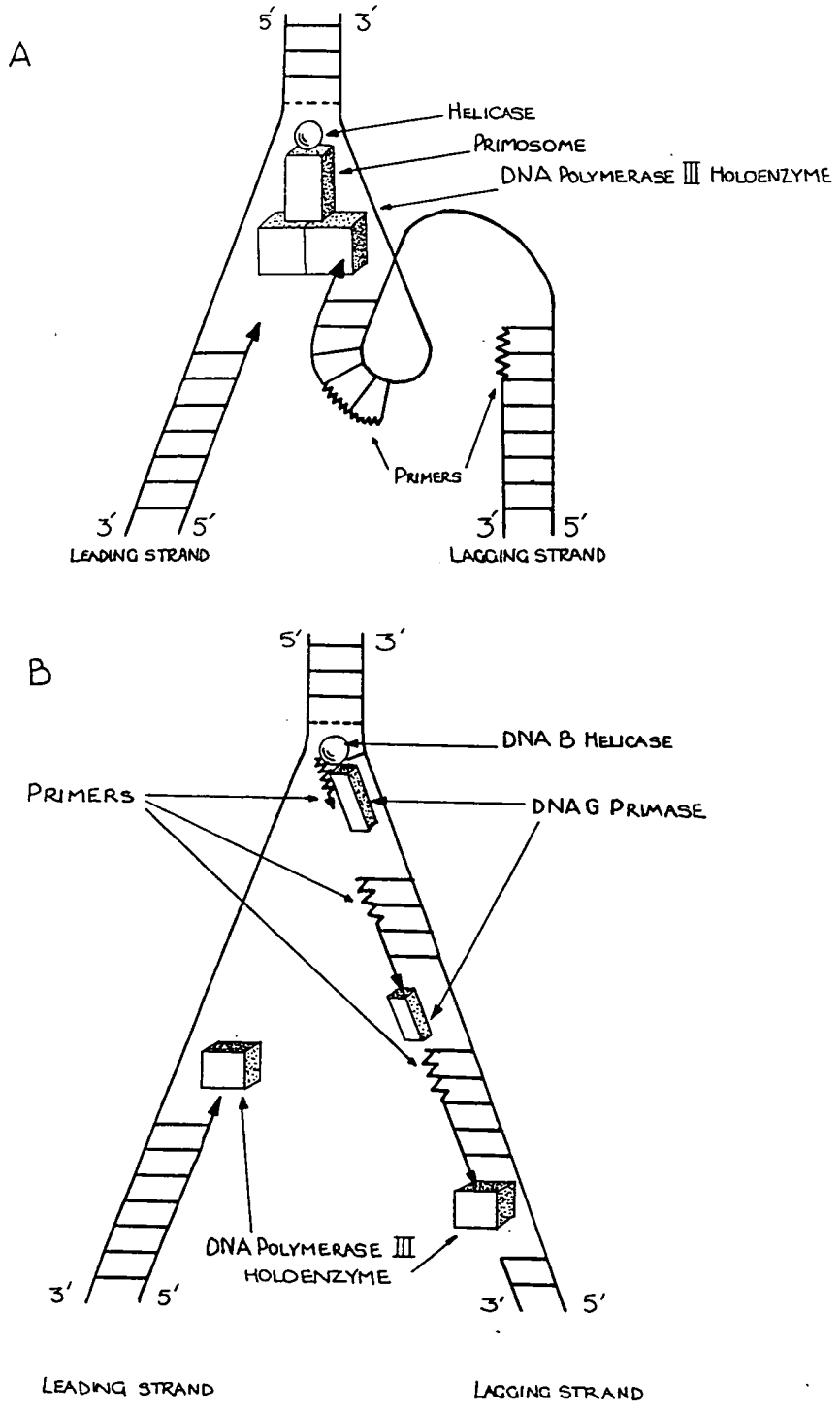
required for initiation from *oriC* as such, but rather is involved only in lagging strand priming as part of the aforementioned 'Replisome' complex (DNA polymerase III holoenzyme and the primosome; Kornberg 1982). In support of this, work by Seufert and Messer (1986) has shown that under certain conditions at least, strand initiation from *E.coli* primosomal assembly sites located near *oriC* can occur.

Kornberg and co-workers (Kornberg 1988, Lasken and Kornberg 1988) obviously favour a model whereby leading and lagging strand synthesis is coordinated via a multiprotein complex consisting of the primosome and the DNA polymerase III holoenzyme (figure 1.1a). Such a structure, together with the looped nature of the DNA template would allow concurrent priming and replication of the lagged strand such that overall DNA replication could proceed in a smooth fashion rather than a jerky sequence of synthesis of one strand followed by synthesis of the other. In favour of this is the fact that the DNA polymerase III holoenzyme appears to be present as two polymerase subassemblies at the replication fork, as is suggested in the above model (Maki *et al.* 1988).

LeBowitz and McMacken (1986) believe that a much simpler assembly of proteins is responsible for coordinating leading and lagging strand synthesis, being comprised only of DnaB helicase, DnaG primase, SSB, and DNA polymerase III holoenzyme. In this model, the DnaB protein migrates processively in the 5'-3' direction along the lagging strand template, acting as a helicase to permit continuous chain elongation by the leading strand DNA polymerase, as well as functioning as a coparticipant with DnaG primase in the synthesis of the multiple lagging strand primers (figure 1.1b). Thus lagging strand priming (and subsequent DNA synthesis) proceeds in advance of that of the leading strand DNA polymerase complex, in contrast to the Kornberg model in which both events occur concurrently and via the same enzyme complex.

In summary then, it is not clear exactly what proteins and multiprotein complexes are involved in the replication of the *E.coli* chromosome. The primosome has been shown to be required for the replication of phage ϕX174 and various *E.coli* plasmids, although it is not clear what the role of this multiprotein complex in host cell DNA replication is. *In vitro* evidence has suggested that it may not be involved in the priming reaction at *oriC*; whether the same is true *in vivo* is not known. Similarly, it is not known whether the primosome is involved in synthesising the RNA primers required for lagging strand initiations in a replisome complex with DNA polymerase III,

FIGURE 1.1 Schemes for the mechanism of action of *E.coli* replication proteins at the replication fork



(A) Model after Kornberg (1988), with concurrent replication of leading and lagging strands by an asymmetric, dimeric polymerase associated with a primose and helicase. **(B)** Model after McMacken et al. (1987) with separate leading and lagging strand synthesis.

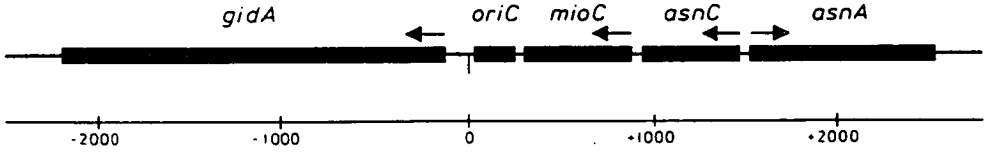
or whether a much smaller collection of proteins can fulfil this role. The question of whether DNA polymerase III acts to sythesise leading and lagging strand DNAs concurrently as part of a replisome complex, or whether these events take place separately, can similarly not be answered at present.

1.2 The origin of replication, *oriC*

1.2.1 The structure of *oriC*

Our understanding of the biochemical events surrounding the initiation of DNA replication has been aided considerably by the identification of several initiation mutants (Wechsler 1978) together with the isolation of minichromosomes which use the *E.coli* origin of replication, *oriC* (Yasuda and Hirota 1977; Messer *et al.* 1978; Zyskind *et al.* 1979). Indeed, the study of initiation represents an excellent example of the achievement brought about by the marriage of classical bacterial genetics with modern *in vitro* biochemical techniques.

FIGURE 1.2 Genetic and physical map of the *oriC* region of the *E.coli* chromosome



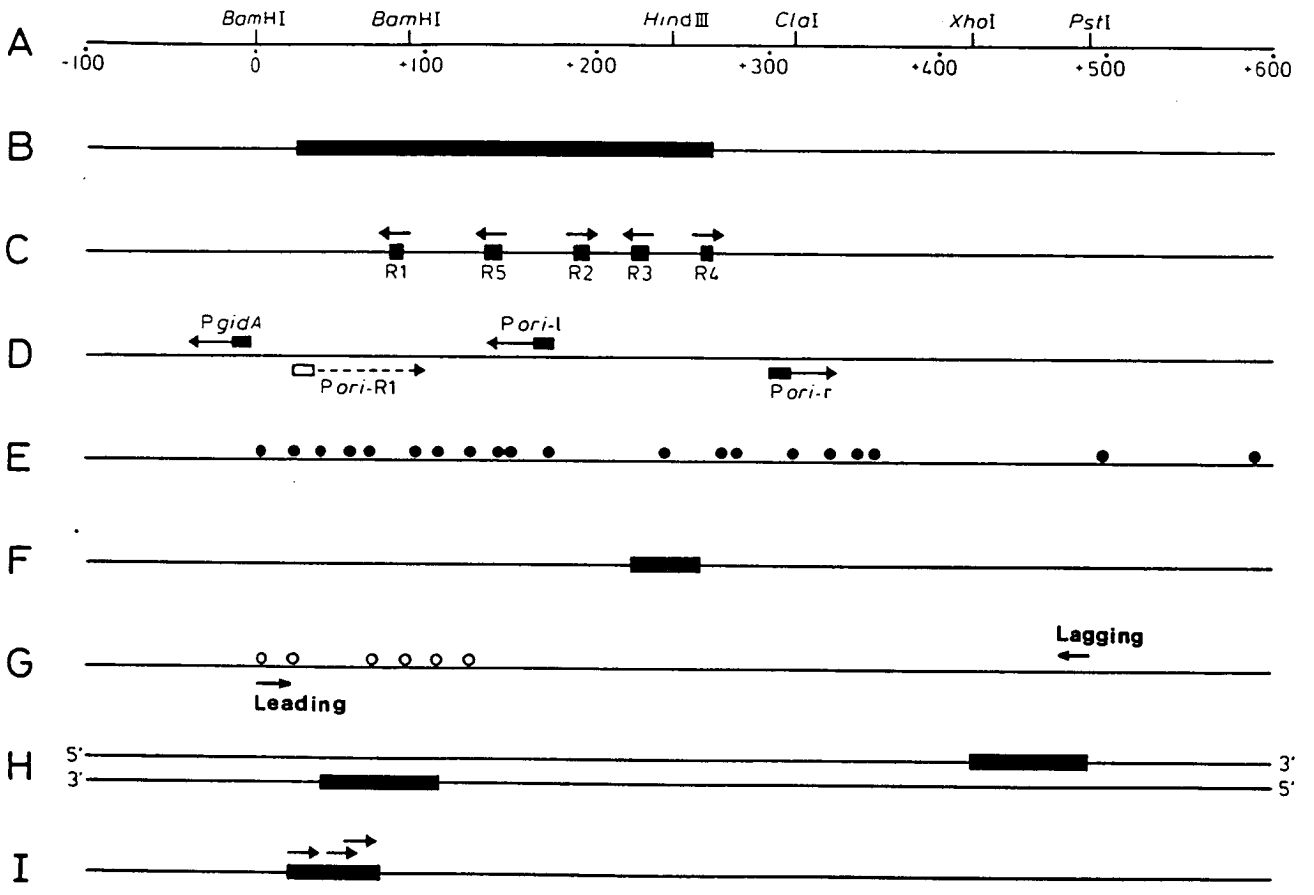
The arrows at the top indicate the directions of transcription. The numbers at the bottom indicate the number of base pairs from position 1, which is defined as the first nucleotide of the BamHI recognition sequence site located immediately counterclockwise (left) of the minimal oriC region. Taken from McMacken et al. 1987.

The origin of replication of *Escherichia coli, oriC*, is located at 83.5 minutes on the genetic map, and is flanked by genes of largely unknown function (although recent evidence suggests that *oriC* may in fact be located within an *asn* operon; Kolling *et al.* 1988). A genetic and physical map of the *oriC* region of the *E.coli* chromosome is shown in figure 1.2. Minichromosomal DNA, which is able to function as an origin of

replication both *in vivo* and *in vitro*, has been narrowed down to a minimal origin of 245bp by use of a joint replicon (Oka *et al* 1980). However, evidence suggests that DNA adjacent to this minimal origin is required for efficient functioning of *oriC in vivo* (Lother *et al.* 1985; Meijer and Messer 1980).

The DNA sequence of the *oriC* region has been determined (Meijer *et al.* 1979; Sugimoto *et al.* 1979), and has revealed several interesting features which may be of importance with regard to its unique function (figure 1.3). There are 11 GATC sequences (the site of methylation by the *dam* methylase (Lacks and Greenberg 1977; Geier and Modrich 1979), and both *in vitro* (Hughes *et al.* 1984, Messer *et al.* 1985) and *in vivo* (Smith *et al.* 1985, Russell and Zinder 1987) experimental evidence has suggested an important role for these sequences in initiation. (the role of these sequences in regulating chromosome replication is discussed in section 1.3.2). Also present in the minimal origin are 4 sites known to bind the DnaA protein (Fuller *et al.* 1984; Matsui *et al.* 1985). These 'DnaA boxes' (R1, R2, R3 and R4) comprise a nine base pair sequence to which DnaA protein binds in a cooperative fashion (Fuller *et al.* 1984). as the first stage (it is postulated) in the initiation process. A strong DNA gyrase binding site has been mapped within the minimal origin between DnaA boxes R3 and R4 (figure 1.3; Jacq *et al.* 1980, Lother *et al.* 1984), and binding to this site may underlie the observed requirement for DNA gyrase in the minimal *oriC* replication system (Kaguni and Kornberg 1984, Lother *et al.* 1984). Three direct repeats of a putative DnaB-DnaC consensus binding sequence can also be found within this minimal origin, and this may represent the site of entry of these proteins, possibly through an interaction with DnaA protein in the initiation process (Bramhill and Kornberg 1988). In addition, there are numerous direct and indirect repeats which could form a variety of secondary structures (Meijer *et al.* 1979). It is likely that the aforementioned features play a crucial role in the ability of *oriC* to direct initiation of replication. The cloning and subsequent sequence analysis of a number of origins from other enterobacteria which can direct initiation in *E.coli* have revealed a high degree of similarity to the *E.coli oriC* (Zyskind *et al.* 1981,1983). Virtually all GATC sequences are conserved, as are the sequences shown to bind the DnaA protein. The importance of these conserved regions has been further demonstrated by detailed mutational analysis (Oka *et al.* 1984). Mutations which impair *oriC* function lie almost exclusively in conserved regions; the most pronounced of these lie in the DnaA binding sites, although some (but not all) mutations in GATC sequences also impair function. Neutral mutations lie in the regions between conserved sequences, although since insertions and deletions in these regions impair *oriC* function, it has been

FIGURE 1.3 Interesting features of the *oriC* region



(A) Map location of selected restriction endonuclease recognition sites. Numbering (+1 position) is from the first nucleotide of the leftmost BamHI site. **(B)** Extent of the minimal replication origin (oriC), with boundaries at nucleotide positions 23 and 267. **(C)** Distribution and orientation of DnaA box sequences. **(D)** Location of putative promoter elements. The arrows indicate direction of transcription. **(E)** Distribution of Dam methylase GATC sites. **(F)** Location of the major DNA gyrase binding site. **(G)** Distribution of the predominant RNA:DNA transitionpoints for the counter-clockwise leading strand synthesis. Arrows show the direction and presumed location of the initiation of leading and lagging strand synthesis. **(H)** Location of the binding sites for a single stranded DNA binding protein isolated from E.coli membrane fractions. **(I)** Location of the three repeating sequences that may represent binding sites for a DnaB-DnaC protein complex. Taken from McMacken et al. 1987.

concluded that the spacing between the conserved regions, as well as the sequences themselves, are of importance for *oriC* function (Oka *et al.* 1982; Asada *et al.* 1982).

1.2.2 The role of RNA polymerase in initiation

Although a great deal has been learned in recent years as regards the events occurring during initiation, utilising both *in vivo* and *in vitro* techniques, our knowledge concerning the exact role of many of its participants remains curiously vague. Most notable of these concerns the role played by RNA polymerase. Various *in vivo* (Lark 1972; Zyskind *et al.* 1977; Bagdasarian *et al.* 1977; Atlung 1981; Tanaka *et al.* 1983) and *in vitro* (Kaguni and Kornberg 1984) studies have implicated RNA polymerase as playing an early role in the initiation of DNA replication. However, whether the RNA polymerase is responsible for synthesising the primer RNA is not known. It has been demonstrated (Kaguni and Kornberg 1984) in an *in vitro* system lacking DNA primase that RNA polymerase can directly prime DNA synthesis at *oriC*. However, similar *in vitro* studies (Ogawa *et al.* 1985) have demonstrated that DNA primase can also fulfil this role, in fact with a much greater efficiency than RNA polymerase. The situation *in vivo* as to which enzyme is responsible can only be guessed at. However, in favour of RNA polymerase acting as the 'priming' enzyme, it appears that *oriC* replication *in vitro* using RNA polymerase for priming is dependent upon RNAase H to maintain template specificity, similar to the situation observed *in vivo* (Kaguni and Kornberg 1984, van der Ende *et al.* 1985). In contrast, *in vitro* priming by DNA primase is independent of RNAase H (Ogawa *et al.* 1985). Additionally, deletion of the DnaB-DnaC binding sites (mentioned in the previous section) appears to impair *oriC* replication both *in vivo* and in the RNA polymerase *in vitro* replication system. Again, priming by DNA primase *in vitro* appears to be unaffected by these deletions (Bramhill and Kornberg 1988). Bramhill and Kornberg have used these findings to argue that the *in vitro* RNA polymerase-primed replication system is probably a more accurate representation of the true situation *in vivo* than is provided by the DNA primase system as regards the initial priming reaction at *oriC*. As a further complication, it has been shown *in vitro* that an RNA polymerase step in initiation precedes binding by the DnaA protein (Kaguni and Kornberg 1984). Whether this represents a pre-priming step involving RNA polymerase followed by priming (for example transcription of an 'initiator gene'), or whether this represents the priming reaction itself is not known.

1.2.3 Transition sites between primer RNA and newly replicated DNA

Recent work (Kohara *et al.* 1985) has demonstrated a remarkable heterogeneity of location for the transition sites between RNA and DNA molecules at *oriC*. By utilising temperature sensitive initiation mutants to achieve synchronous cultures, Kohara *et al.* have shown that at least nine transition sites occur both in and around the minimal origin (Figure 1.2). On the counter-clockwise (R) strand a marked

clustering of transition sites exists towards the leftmost part of the minimal origin; while on the complementary strand no transition sites within the minimal origin are found, although several are to be found in the upstream flanking region.

The authors use their results as evidence toward the unidirectional initiation model proposed by Hirose *et al.* (1983). This model suggests that DNA synthesis first begins in the counter clockwise direction on the R strand from one of the transition sites in *oriC* (i.e. from left to right in figure 1.2). This is followed by intermittent lagging strand synthesis (primed by DnaG primase) from the transition sites located at multiples of 400bp downstream from the original R strand initiations (i.e. replication in the leftward direction from the transition sites located in the +4–500 bp region shown in figure 1.2). This is consistent with the finding that the replication fork proceeds counter clockwise during the earliest period of replication (Yoshimoto *et al.* 1986), although against this Tabata *et al.* (1983) have presented evidence that *oriC* directs simultaneous bidirectional replication. Interestingly, the majority of RNA:DNA transition regions occur near to GATC methylation sites (figure 1.2), although whether this is significant or merely a manifestation of the large number of GATC sites in this region remains to be determined. In addition, the position of these transition sites coincides well with the termination sites of counter clockwise transcripts entering *oriC* from outside promoters both *in vivo* and *in vitro* (Rokeach and Zyskind 1986), leading onto the possibility that transcription from outside of *oriC* may play a role in determining the frequency of initiation.

1.2.4 Promoters in and around *oriC* –the role of the *mioC* gene

An obvious step towards clarifying the role of the RNA synthetic events in initiation would be an analysis of the promoters responsible for such transcription. A promoter found within the minimal origin, P*ori-L* (Lother and Messer 1981), has been shown to lead to counter-clockwise transcription through *oriC* in an *in vitro* system, and its position slightly upstream of the majority of the RNA:DNA transition sites on the R strand would suggest it is a strong candidate for an 'Initiation Promoter' (figure 1.2b). However, activity of this promoter *in vivo* has not been demonstrated (Junker *et al.* 1986).

DNA in the regions flanking *oriC* may play an important part in the early RNA polymerase mediated step in initiation. *oriC* is located in the intercistronic region between the genes coding for the 70kd and 16kd proteins (*gidA* and *mioC* respectively; von Meyenburg and Hansen 1980, von Meyenburg *et al.* 1982) (figure 1.3). The role of these gene products in *E.coli* metabolism is unclear, although the subtle

phenotype of *gid* mutants (glucose inhibition of division) has implicated the former as being involved in cell division (von Meyenburg *et al.* 1982). The function of the MioC protein (formerly known as the 16kd protein) is undetermined. Both genes are transcribed in the same direction; the *gidA* transcript reading away from *oriC*, and the *mioC* transcript reading towards it (Buhk and Messer 1983; figure 1.2).

A variety of data suggests that transcription of the genes adjacent to *oriC* may influence initiation. *In vivo* work (Junker *et al.* 1986; Kohara *et al.* 1985) has demonstrated that transcripts from the *mioC* promoter enter and terminate at sequences within *oriC*, and RNA polymerase has been shown to pause at the same sites *in vitro* during transcription (Rokeach *et al.* 1987). More detailed work on the 3' ends of these transcripts (Rokeach and Zyskind 1986) has lead the authors to claim a correlation between these ends and the DNA:RNA transition sites previously identified by Kohara *et al.* (1985). They further suggest that the *mioC* transcript might well represent the primer RNA used in leading strand initiation.

However, Rokeach and Zyskind identified at least 20 different strong termination sites together with a variety of weaker ones, with only nine of these corresponding to RNA:DNA transition sites. Since these termination sites are all located within the 245bp minimal origin (i.e. on average 1 for every 10 base pairs), a random distribution might perhaps justifiably be claimed. At best a correlation between GATC sequences and termination sites might be considered. Indeed, the evidence of Rokeach and Zyskind is in direct disagreement to that of Kohara *et al* (1985), who stated that transcript termination of the *mioC* gene did not coincide with major R-strand RNA:DNA transition sites. However, they did point out a certain degree of correlation with GATC sites.

Whether or not transcription of the *mioC* gene represents the 'primer' transcript in initiation, a variety of additional data points to it being involved in the process. Deletion of the *mioC* promoter or its adjacent DnaA box leads to a reduced copy number of *oriC* minichromosomes (Stuitje *et al.* 1986, Lobner-Olesen *et al.* 1987). Additionally, transcription from the *mioC* promoter is negatively regulated by the DnaA protein and depends upon *dam* methylation for maximal activity (Lother *et al.* 1985, Lobner-Olesen *et al.* 1987). Both of these activities are known to influence initiation from *oriC* (see sections 1.3.2 and 1.3.4), so a model whereby the *mioC* transcript acts as the 'primer' in initiation, with regulation mediated via this transcript has obvious attractions.

However, it should be remembered that DnaA protein acts in a positive fashion

in controlling the frequency of initiation (Atlung *et al.* 1987); the fact that it represses transcription from *mioC* into *oriC* would tend to argue against these transcripts being the primer RNA. Furthermore, replication of *oriC* plasmids can proceed both *in vivo* and *in vitro* without the *mioC* gene being present (Oka *et al.* 1980), indicating that it certainly is not an absolute requirement for initiation as would be expected of the 'primer' transcript. However, it should be appreciated that although such replication can take place, it occurs with a reduced efficiency (Meijer and Messer 1980), suggesting that the *mioC* transcript might be the primer of choice, although other transcripts may be able to fulfil the role if required. Such a situation possibly bears comparison with the replication of ColE1-type plasmids. In this instance a region of DNA upstream of the plasmid origin which is ordinarily required for replication can, under some circumstances be deleted (Gayle *et al.* 1986). This region codes for a small RNA species, RNA II, 550 bp in length, which is transcribed from an upstream promoter into the plasmid origin (Tomizawa *et al.* 1977). (For more details about the replication of ColE1 plasmids see chapter 7). Transcription of this RNA species, and subsequent plasmid replication, is rifampicin sensitive, thus implicating RNA polymerase as the enzyme responsible (Clewell *et al.* 1972; Sakakibara and Tomizawa 1974). Gayle *et al.* (1986) have demonstrated that deletion of this RNA species is possible without preventing plasmid replication, readthrough into the plasmid origin from an adjacent promoter being able to substitute for the RNA II transcript. Indeed, the copy number of the plasmid increased tenfold, presumably due to the deletion removing the small RNA molecule RNA I, normally required for negative regulation (Tomizawa and Itoh 1981). If indeed the *mioC* gene fulfils the same role in *E.coli* replication as does RNA II in ColE1 replication, this might explain how minimal origins lacking flanking DNA are able to replicate.

What is the role in initiation of the other genes which flank *oriC*, the *gidA*, *asnC* and *asnA* genes? Some fascinating data which explores the relationship between these genes, whilst additionally strengthening the case for *mioC* being an initiation gene has recently become available (Kolling *et al.* 1988; Gielow, Kucherer, Kolling and Messer, Mol. Gen. Genet. in press). By a combination of S1 nuclease mapping and *in vitro* transcription-translation assays, it would appear that these genes form part of an operon concerned with asparagine metabolism, apparently unconnected with DNA replication. In summary, it seems that the *gidA*, *asnC* and *asnA* genes are all subject to negative regulation by the AsnC protein, while the *mioC* gene is not. Additionally, the majority of *asnC* transcripts appear to traverse the replication origin, in contrast to those originating at *mioC* which stop within the origin region (and thus presumably would be available to act as primers). While the level of

asnC transcripts entering *oriC* did appear to be reduced with increasing levels of DnaA protein, this was due to a termination of transcription downstream of the *asnC* promoter at the *mioC* DnaA box rather than any direct repression of *asnC* promoter activity. Thus this result is not incompatible with the model that the surrounding genes are subject to a different form of control than *oriC*. Gielow *et al.* use this to suggest that *oriC* and *mioC* may at some time during the evolution of *E.coli* have transposed into an *asn* operon, and cite in support of this a comparison with the *B.subtilis* chromosome, in which the replication origin is located in close proximity to various genes involved in DNA metabolism (von Meyenburg and Hansen 1987). In contrast, in *E.coli* these two regions are separated by approximately 40kb of DNA (Ogasawara *et al.* 1985).

In summary then, it seems likely that transcription of the *mioC* gene adjacent to *oriC* probably does play an important role in the initiation process, although exactly what this role is remains undetermined. Some evidence suggests that it may represent the primer transcript, although equally so a variety of evidence suggests that its role is a subsidiary one. Transcription of this gene may be an important 'first stage' in initiation (prior to priming), or it may in fact represent the priming stage itself. If the latter is indeed true, it is uncertain whether this occurs before, during or after any DnaA-mediated protein event at *oriC*.

1.3 The Regulation Of Initiation

1.3.1 Introduction -models for the control of initiation

One of the major problems regarding the regulation of initiation is in determining whether a potential controlling factor is really determining the frequency of initiation, or whether it simply effects the efficiency at which the whole process occurs.

As yet, no particular element has been demonstrated to play a decisive role in determining what causes cells to begin replicating their DNA. At least three processes have been implicated: methylation of the origin DNA, stringent control, and DnaA protein levels; but whether these represent controlling elements or are simply affecting the efficiency of the initiation event cannot be determined. Whether the triggering of initiation is the responsibility of a single molecule, itself part of a much larger regulatory system, or whether a whole host of interacting elements exert their effect simultaneously at *oriC* to trigger the initiation event is also unknown.

Control systems involving a single molecule as being responsible for initiation control have been proposed, including an initiator accumulation model (Jacob *et al.* 1963; Helmstetter *et al.* 1968; Sompayrac and Maaloe 1973; Margalit *et al.* 1984), and an inhibitor dilution model (Helmstetter *et al.* 1968; Sompayrac and Maaloe 1973; Margalit *et al.* 1984).

The first of these models proposes the accumulation of an autoregulated 'initiator', synthesis of which is coupled to mass increase, until such a time as a sufficient number of molecules of this 'initiator' have accumulated to allow replication to begin. For such a model to work would require that this 'initiator' molecule be consumed during the act of initiation.

The other model suggests the presence of a constitutively expressed unstable inhibitor molecule (Pritchard *et al.* 1969; Messer *et al.* 1975; Pritchard 1978, 1984). Since the intracellular level of this molecule would be dependent upon gene dosage, as the cell increased in size, the repressor would be diluted out until a critical point were reached whereby initiation could occur, the repressor gene would be replicated (it would be assumed to be located near the origin) and the intracellular repressor level would be restored until the next mass doubling at which time initiation could occur again. This model elegantly explains how faster growing (and hence larger) cells would achieve nested replication forks; the larger the cell, the more origins would be required to maintain a constant inhibitor (and hence *oriC*) concentration.

1.3.2 Role of methylation in the regulation of initiation

A possible role for methylation in *oriC* function came under consideration following the discovery of a large number of GATC sequences in and around the minimal origin (Meijer *et al.* 1979; Sugimoto *et al* 1979). The *E.coli dam* methylase methylates the adenine residue in the GATC sequence (Lacks and Greenberg 1977; Geier and Modrich 1979), and all sites in *E.coli* are methylated (Razin *et al.* 1980). In addition, it is interesting to note that a potential DnaA binding site is present near the promoter of the *dam* gene, and expression from this promoter has been reported to be increased upon thermal inactivation in a *dnaAts* strain (Herman *et al.* 1984). Furthermore, expression of the *dnaA* gene appears to be affected by the level of methylation of the surrounding region (Braun and Wright 1986). These findings lead to an investigation into the role of methylation in the control of DNA replication in *E.coli*, and the evidence which suggests that it might have a role is briefly summarised below.

Transformation efficiencies of *oriC* plasmids into *E.coli* strains deficient in the *dam* methylase have been shown to be greatly reduced compared to *dam*$^+$ strains (Smith *et al.* 1985; Messer *et al* 1985). In addition, replication of unmethylated *oriC* DNA *in vitro* has been reported to occur less efficiently than with a methylated template (Smith *et al.* 1985; Messer *et al.* 1985), although the requirement for methylation *in vitro* appears to be less stringent than *in vivo* (Messer *et al* 1985). Perhaps most interestingly of all, hemimethylated plasmid DNA appears to be incapable of replicating in a *dam* mutant strain (Russell and Zinder 1987), although unmethylated DNA transforms at a high frequency (Messer *et al.* 1985, Russell and Zinder 1987). This suggest a mechanism preventing replication of newly synthesised origin DNA under normal cellular conditions, with the intriguing possibility that the time required to methylate the newly synthesised DNA might be controlling the frequency of initiation at *oriC*. Since unmethylated origin DNA seems able to replicate in a *dam*$^-$ host, it would seem that it is the hemimethylated status of the DNA rather than the absence of methylation which is preventing replication, which could explain the otherwise awkward observation that such strains are viable even though unable to methylate their own DNA (that is, the hemimethylated status of the DNA is important in preventing replication rather than methylation in itself being necessary for replication to occur).

Of further interest in the methylation story is the report by Ogden *et al.* (1988) that *E.coli oriC* DNA binds to cell membranes only when hemimethylated. This is an attractive finding in that it explains how chromosome segregation can occur: immediately after replication of *oriC*, this region binds to the cell membrane and is the mechanism by which newly replicated origins are separated as the cell grows. It may be this binding of the hemimethylated *oriC* DNA to the cell membrane which in fact prevents its replication; following full methylation the *oriC* DNA is released from the membrane and replication is again possible. In support of this model is the observation that the interval between replication events is decreased in a Dam overproducing strain, as would be expected if the hemimethylated state of the DNA were preventing replication.

However, although this model is superficially highly attractive, it contains a number of unresolved discrepancies. In a *dam*$^-$ strain replication should occur at a much higher frequency than in the wild type, since the DNA is never hemimethylated and should therefore be available to initiate immediately following replication. This is not the case, the frequency of initiation being unchanged in a *dam*$^-$ strain (Messer *et al.* 1985, Russell and Zinder 1987). Additionally, if hemimethylation were vital for

membrane binding (and hence chromosome segregation), *dam*$^-$ strains should be inviable by virtue of being unable to segregate their DNA. Again this is not found. Finally, the time taken to fully methylate *oriC* DNA following replication is only about 10 minutes or so (Ogden *et al.* 1988), far less than the minimum period between successive rounds of initiation in even the fastest growing cultures.

It would appear likely then, that rather than acting as a controlling element determining the *frequency* of initiation *per se*, the state of methylation at *oriC* probably determines the *efficiency* with which initiation can occur, an important distinction. However, given the importance of methylation in eukaryotic systems as a means of gene regulation it would not be altogether surprising if its role in prokaryotes were more important than previously supposed.

1.3.3 Effect of the stringent response on the regulation of initiation

The stringent response occurs when cells are starved of one or more limiting amino acids (cf. Cashel 1975; Gallant 1979). When the level of any amino-acyl t-RNA becomes limiting the stringent response is initiated; this is mediated through the nucleotide ppGpp and leads to a decrease in the synthesis of certain RNA species. Since initiation in *E.coli* requires an RNA synthesis event (Zyskind *et al.* 1977), the possibility that this event might be subject to stringent control exists.

Replication of the ColE1-type plasmid pBR322 has been demonstrated to be inhibited upon induction of the stringent response (Hecker *et al.* 1983; Lin Chao and Bremmer 1986). Similarly, work by Seror *et al.* (1986) involving *B.subtilis* has demonstrated that induction of the stringent response prevents any new initiation of DNA synthesis for at least 30 minutes. They also reported that preliminary studies using *E.coli* had produced the same result.

The question remains as to what role, if any, the stringent response performs during normal steady-state growth of the cell. Although the cell size (and therefore the number of origins) is altered with the growth conditions, stringent control is a specific response to amino acid starvation; as such it should not play a role in normal cellular metabolism. Therefore it is hard to see how it could influence the frequency of initiations unless under exceptional circumstances. Rokeach and Zyskind (1986) have claimed that expression of the *mioC* gene adjacent to *oriC* is stringently controlled, and suggest this implicates the stringent response in the regulation of initiation. Moreover, it has been reported (van Verseveld *et al.* 1984) that at slow growth rates the level of ppGpp negatively regulates cell division. However, a direct

involvement of either the *mioC* gene or the stringent response in determining the frequency of initiation has not been demonstrated. At best it can be said of the stringent response is that its effect resembles that of methylation; it can influence the efficiency of the initiation event but not necessarily the frequency at which it occurs.

1.3.4 Role of the DnaA protein in initiation control

Both of the models previously mentioned explain control of the initiation event by postulating the existence of a specific 'initiation' factor, acting in either a positive or negative capacity to regulate initiation. The isolation of temperature sensitive mutants which affected initiation exclusively, and the fact that these mutations all mapped to the same map location, the *dnaA* gene, has lead to speculation that this gene product might in fact be this 'initiation' factor (Atlung *et al.* 1985b, Atlung *et al.* 1987).

That the DnaA protein can act in a regulatory manner is without a doubt (Atlung *et al.* 1985a; Braun *et al.* 1985; Lother *et al.* 1985; Wang and Kaguni 1987); similarly it is known to bind to, and form large initiation complexes with *oriC* (Fuller *et al.* 1984). In addition, it is absolutely required, both *in vivo* and *in vitro* for initiation to occur (Tomizawa and Selzer 1979; Fuller *et al.* 1981, 1983; Kaguni *et al.* 1982; Fuller and Kornberg 1983). Is the DnaA protein the initiator molecule then? It is certainly known to act at an early stage in the initiation process, preceded only by the RNA polymerase mediated step (Kaguni and Kornberg 1984). In addition, it fulfils one of the criteria demanded by the Pritchard Repressor model in that it is located very close to *oriC*, being located a mere 42kb counter-clockwise of *oriC* and is thus one of the first genes to be replicated following initiation (Hansen and von Meyenburg 1979; Miki *et al.* 1979). However, despite the wealth of data which has accumulated in recent years concerning DnaA, its role in initiation (whether it is a regulatory factor or simply necessary for the event to occur for example) is still far from clear.

The *dnaA* gene codes for a mildly basic protein of 52.5kd as determined by DNA sequence analysis (Hansen *et al.* 1982; Ohmori *et al.* 1984). As well as being required for initiation from *oriC*, the DnaA protein has been shown to be required for replication of plasmids P1 and F (Hansen and Yarmolinsky 1986), RK2 (Gaylo *et al.* 1987), and pSC101 (Frey *et al.* 1979). It is unclear whether DnaA is required for the replication of plasmids R1 and ColE1 (Nagata *et al.* 1988, Molin and Nordstrom 1980, Seufert and Messer 1987, Frey *et al.* 1979, Hansen and Yarmolinsky 1986). Furthermore, DnaA protein levels appear to affect the transposition frequency of Tn5 (which has a DnaA binding site; Yin and Reznikoff 1987), although it is unclear whether

this is a direct effect of DnaA or mediated via some intermediary action (such as affecting the level of *dam* methylation for example). DnaA is a DNA binding protein, and binds to a specific sequence (DnaA boxes), which are present at *oriC* and at the origins of several *E.coli* plasmids (and to which binding has also been demonstrated; Fuller *et al.* 1984). At *oriC* at least, this binding appears to be cooperative, with a complex of about 20-40 molecules of DnaA protein being involved (Bramhill and Kornberg 1988). DnaA is an ATPase; it binds ATP with high affinity, with the ATP being hydrolysed to ADP in a DNA-dependent manner, possibly during the duplex opening stage of initiation at *oriC* (Sekimizu *et al.* 1987). All forms of the DnaA protein appear to be able to bind to DNA, although only the ATP form seems active in initiation (Sekimizu *et al.* 1988). This may represent a means of modulating the frequency of initiation; determining the activity of the initiation factor rather than its presence *per se*. It has recently been reported that cAMP interacts with the ADP-DnaA complex to release DnaA protein, thus making it available for interaction with ATP once more (Patrick Hughes, Ahmed Landoulsi and Masamachi Kohiyama, submitted to Cell 1988). If so, this would implicate cAMP levels as being an important determinant of the initiation frequency in *E.coli*. However, it has also been reported that cardiolipin, a diacidic membrane phospholipid is also able to restore the inactive ADP-DnaA to the active ATP-DnaA form (Sekimizu and Kornberg 1988). This has the attraction that it provides a link between chromosome replication and the cell membrane. However, whether either or both of these mechanisms are important in regulating the levels of the active ATP form of DnaA *in vivo*, or whether they are artifacts of an *in vitro* replication system cannot be ascertained. However, they do add further complications to what is already a highly complex picture.

DnaA protein was first implicated as a possible controlling factor in DNA initiation following an investigation into the properties of the *dnaA46ts* allele (Hirota *et al.* 1968). This mutant can initiate replication and exhibits balanced growth at 28°C. However, as the temperature is increased, the amount of DNA per unit mass decreases, suggesting a decreasing activity for the DnaA46 protein (Hansen and Rasmussen 1977). Thus the availability of active DnaA protein appeared to act as a limiting factor in DNA replication.

A regulatory role for the DnaA protein was further suggested by the behaviour of an intragenic suppressor of *dnaA46, dnaAcos* (Kellenberger-Gujer *et al.* 1978). This mutant was isolated by its ability to grow at 42°C in contrast to the *dnaA46* parental strain. When shifted back to 30°C, an increase in the initiation frequency of chromosome replication was observed, as if the DnaAcos protein was exhibiting extra

activity as compared to both the DnaA$^+$ and DnaA46 proteins. Reversion frequencies of *dnaAcos* to *dnaA46* suggested that *dnaAcos* was in fact a double mutant; the original *dnaA*46 mutation with an additional intragenic mutation which resulted in the *dnaAcos* phenotype. (Sequencing of the *dnaAcos* allele at a later date revealed that it in fact possessed 3 point mutations compared to the *dnaA*$^+$ gene; Braun *et al.* 1987).

This phenotype could be explained in two ways:

- The secondary mutation could have increased the activity of the DnaA46 protein such that at 42°C the loss of function caused by the original mutation was compensated for by the secondary mutation, this compensation being too great at 30°C, or alternatively,

- The temperature resistance in the *dnaAcos* strain results from over-production of the DnaA46 protein, compensating for a reduction in activity at 42°C, but leading to an excess of fully functional protein at 30°C.

(Later work (Braun *et al.* 1987) has in fact suggested that the former of these is probably the more likely explanation). Whatever the cause of the *dnaAcos* phenotype, two important conclusions could be drawn:

1. The amount or activity of the DnaA protein appeared to regulate the frequency of initiation, making it a prime candidate for the role of 'initiation factor'.

2. The DnaA protein must have at least two active sites or functions, a different one being affected by each of the aforementioned mutations, leading on to the idea that *dnaA* is autoregulated; the *dnaA46* mutation affecting the ability of the protein to act in initiation perhaps, the *dnaAcos* affecting its ability to suppress its own synthesis for example.

These ideas were encapsulated by Hansen and Rasmussen (1977), who proposed that the DnaA protein had 2 functions; a positive one, essential in initiation of replication, and a negative one as a repressor of its own synthesis. This hypothesis was at least partially supported by the behaviour of *dnaAts* mutants, which, after incubation at 42°C and subsequent return to the permissive temperature, were found to have excess capacity for initiation of replication (Orr *et al.* 1978; Frey *et al.* 1981; Fralick 1978). This capacity was unaffected by chloramphenicol, demonstrating that *de novo* protein synthesis was not required and that the DnaA46 protein is heat re-naturable (Evans and Eberle 1975; Messer *et al.* 1975; Hanna and Carl 1975). This pattern of behaviour would agree with that predicted by the Hansen-Rasmussen model: at the restrictive temperature the DnaA46 protein would be denatured, presumably losing its autorepression function in addition to its ability to act in initiation. As a result, de-repression of the *dnaA* gene would occur, and upon return to the permissive

temperature an excess of now functional DnaA protein would be present, leading to over-initiation.

Autorepression of *dnaA* gene expression has since been demonstrated (Atlung *et al.* 1985a; Braun *et al.* 1985, Wang and Kaguni 1987, Kucherer *et al.* 1986, Hansen *et al.* 1987), although whether it is *the* positive control factor in initiation is perhaps less proven. One of the main problems in elucidating the role of the DnaA protein in initiation is in the pleiotropic nature of its effects. In addition to regulating its own expression, it has been shown to negatively affect transcription of the *ftsZ* gene (Masters, Paterson, Popplewell, Owen-Hughes, Pringle and Begg 1988, submitted to Mol. Gen. Genet.), the *pheA* gene (T.Atlung 1984, citing unpublished results), and affect *trp* operon attenuation (Atlung and Hansen 1983). In addition, it has been shown to repress expression of the *mioC* gene located immediately adjacent to *oriC* (Lother *et al.* 1985; Rokeach and Zyskind 1986) and which could act as the primer RNA in leading strand initiation. However, given that DnaA is known to bind to, and form complexes with *oriC* (Kaguni and Kornberg 1984), it is unlikely that its involvement in initiation control is confined merely to regulating the expression of genes whose products may themselves be involved in the process.

It is possible that eventual control over initiation might involve a balance between both positive and negative control elements of the DnaA protein. The DnaA protein might negatively control the initiation event by repressing expression of the *mioC* gene, and positively by acting in an otherwise undefined manner in complex with *oriC*. Thus its mode of action could be thought of as being similar to the λcI protein, which is able to act as both a positive and negative regulator of gene expression depending on the circumstances. This could perhaps go some way towards explaining the contradictory nature of some of the results concerning over-expression of *dnaA*.

Original studies concerning the effect of over-expression of the *dnaA* gene following its induction under control of the *lac* promoter came to the conclusion that it was not a limiting factor in the control of initiation, since no effect on the level of DNA was observed (Churchward *et al.* 1983; Bremer and Churchward 1985), although they did observe a reduction in the growth rate of some strains. However, since they only measured DNA concentration on a gross scale, any small changes due to transient over-initiation would not have been detected. When this was examined in detail by Atlung *et al.* (1985b, 1987), they found that increased intracellualar levels of DnaA did indeed result in over-initiation, but these extra forks were aborted close to

the origin, and therefore not increasing significantly the overall DNA content. However, the question as to why these forks are aborted when those caused by over-initiation in the *dnaAcos* mutant are able to continue must be asked (Kellenburger-Gujer *et al.* 1978). Recent work by Xu and Bremer (1988) came to the conclusion that earlier data (Churchward *et al.* 1983, Bremer and Churchward 1985) suggesting that excess DnaA had no effect on the initiation frequency were incorrect, since the oversupply of DnaA from a *lacUV5* promoter did not in fact take place due to growth of the strains in rich medium. When these experiments were repeated in minimal media, efficient over-expression of *dnaA* gene was found to be possible. Xu and Bremer concluded that oversupply of DnaA did cause over-initiation, leading to twice the normal number of chromosomes in the cell. This agrees with the hypothesis that DnaA acts to stimulate initiation, although it is in disagreement with the multiple aborted initiation forks reported by Atlung *et al.* (1985a)(in which the total amount of DNA in the cell was little changed).

Several groups, amongst them Atlung *et al.* (1985a, 1987), obviously favour the DnaA protein as the positive factor involved in initiation regulation. However, despite several attractive lines of evidence for this model, a number of important discrepancies exist. A major influence in the formation of this model was the excess initiation potential which accumulated upon incubation of a *dnaAts* strain at the restrictive temperature. This was thought to be due to accumulation of thermally inactive DnaAts protein, which was renatured upon return to the permissive temperature, allowing excess initiation because of an abnormally high amount of the protein being present. However, work by Sakakibara and Yuasa (1982), in which they examined DnaA protein levels *in vivo* by means of two dimensional protein gel electrophoresis, showed that the thermosensitive DnaA protein was actively *degraded* at 42°C. After 60 minutes at the restrictive temperature, the amount of protein was less than a quarter the amount before the temperature shift. This would strongly disagree with the aforementioned model to explain accumulation of initiation potential. Sakakibara and Yuasa further demonstrated that synthesis of DnaA protein proceeded uniformly throughout the cell cycle, concomitant with the increase in cellular mass, not the behaviour expected if gene dosage effects played an important part in regulating *dnaA* and through it, initiation control. On the basis of their results, the authors question the role of the DnaA protein in regulation of initiation, suggesting that since *dnaAts* strains were able to accumulate initiation capacity with three quarters of their DnaA protein absent, then cells must normally maintain an excess of this protein. This is somewhat at odds with the model envisaged for DnaA function by Atlung *et al.* (1985a). Work in which DnaA protein levels in the cell have been examined by immunoblotting has suggested that

cells maintain roughly 800–2100 (Sekimizu *et al.* 1988) or 500 (this work, chapter 5) molecules per cell, which does indeed appear to be a large excess over that required for initiation at *oriC* (20–40 molecules; Bramhill and Kornberg 1988). This would further argue that the amount of DnaA protein is not normally a limiting factor in initiation control. However, it is possible that DnaA protein, although in excess, may not be in a form available to take part in initiation under most conditions and thus could act as a limiting factor in this way (for example it might normally be bound to DnaA boxes located around the *E.coli* chromosome, of which about 2000 or so exist (M. Masters pers. comm.) or additionally it could be held in an inactive ADP–DnaA complex form).

What are the possibilities that *DnaA* protein performs only a minor role in the actual regulation of initiation? Mutants of *rpoB* which result in over-initiation have been reported (Rasmussen *et al.* 1983; Tanaka *et al.* 1983), and since an RNA polymerase step precedes involvement of the DnaA protein in initiation (Zyskind *et al.* 1977; Fuller and Kornberg 1983), it is possible that RNA polymerase is in some way involved. However, since *rpoB* mutants are able to suppress temperature sensitive *dnaA* alleles (Atlung 1981), over initiation in the aforementioned *rpoB* strains may simply be due to an indirect effect on DnaA. Experiments involving a block to new protein synthesis in a *dnaAts* strain, followed by a return to the permissive temperature following thermal inactivation, resulted in a transient stimulation of initiation, suggesting the possible involvement of a negative control factor in regulation (Tippe–Schindler *et al.* 1979). If this were so, this factor would presumably have to be relatively unstable. However, only circumstantial evidence as to the existence of such a negative control protein exists at present.

1.3.5 Summary

It can probably be appreciated that the regulation of initiation of *E.coli* DNA replication is a far from simple process. Factors which appear to be involved include methylation, the stringent response, DnaA protein levels (and its activity), and possibly DNA sequences adjacent to *oriC* (e.g. the *mioC* gene). The picture is further complicated by the interrelated nature of many of these control systems. (For example, methylation of DNA directly affects its ability to replicate, but it also affects the level of expression of the *dnaA* gene, which itself can regulate both DNA replication and the expression of the *dam* and *mioC* genes (as well as regulating its own expression by means of a feedback loop). Further complications are introduced depending on whether the DnaA protein is in an 'active' (i.e. complexed with ATP) state

or not.)

The importance of transcription of the *mioC* gene to initiation is similarly unclear. Is it the first stage in the process, or is the binding of the DnaA protein to *oriC* the signal to begin initiation? Does the DnaA protein bind to *oriC* throughout the cell cycle, only causing initiation under certain conditions, or does it only bind when initiation is required? It would seem clear at least that factors other than the DnaA protein must play an important role in regulation. For example, increasing the number of origins results in a titration of DnaA protein levels (presumably by the binding of DnaA to *oriC* DnaA boxes), leading to increased *dnaA* gene expression (Hansen *et al* 1987). For runaway replication not to occur would obviously argue strongly for the involvement of a variety of additional controlling elements. A more understandable picture of the complex process of the regulation of initiation must await considerably more work on the subject it would seem.

Additionally, it is perhaps wise to consider what the phrase 'regulation of initiation' actually means. In essence, it concerns the means by which the timing of initiation is controlled under normal conditions. Alterations to some of the above elements can be seen as affecting the frequency or efficiency of the initiation event; however, this need not necessarily mean that they control the frequency of initiation under conditions of normal growth. For example, over-production of DnaA may lead to extra initiations; this does not automatically mean that DnaA controls the timing under normal conditions, simply that extra DnaA causes over-initiation. DnaA protein may be the means by which the signal to initiate is transmitted, but it still requires the 'clock' to regulate when that signal should be sent.

1.4 The biochemistry of the initiation process

Since the main subject of this thesis is the mechanism by which over-expression of the *groE* genes is able to suppress temperature sensitive mutations in the *dnaA* gene, a description of the biochemical processes involved in initiation should help towards an understanding of how such suppression might be occurring.

1.4.1 RNA polymerase and initiation

Both *in vivo* (Zyskind *et al.* 1977) and *in vitro* (Kaguni and Kornberg 1984) evidence has suggested that RNA polymerase acts at the earliest stage yet identified in initiation. This may involve laying down the primer RNA; alternatively it may be involved in transcribing an 'initiator' gene immediately prior to the initiation event, but

then taking no further part in the process; finally, its role may simply be to separate the DNA strands to allow entry of proteins such as DnaA, DnaB and DnaC which then allow initiation to proceed. At this point it is perhaps interesting to consider what 'instructs' RNA polymerase to perform its role in initiation, whatever it might be. Is the transcription performed continuously throughout the cell cycle, relying on subsequent events (for example involvement of the DnaA protein) to control whether initiation actually takes place, or might changes to repressors, methylation, or possibly the aquisition of an 'initiation sigma factor' specific for *oriC* promoters allow the onset of this transcription at a specific time?

In vivo experiments (Zyskind *et al.* 1977) concerning the role of RNA polymerase examined the effect of adding the antibiotics rifampicin or streptolydigin to a *dnaAts* mutant after growth at the restrictive temperature. This was to allow the aquisition of initiation potential; that is, the ability to initiate DNA replication in the absence of protein synthesis upon return to the permissive temperature. Any effect noted was not therefore due to a simple inhibition of protein synthesis. The rifampicin treatment resulted in a complete inhibition of initiation, whereas streptolydigin did not. Since both antibiotics affect RNA polymerase function, this result might at first seem somewhat contradictory. However, while streptolydigin inhibits chain elongation, rifampicin acts to prevent both the initiation of transcription and its termination, causing increased readthrough of terminators (Newman *et al.* 1982). Since it has already been mentioned that RNA polymerase pauses at a variety of sites within *oriC* during transcription of the *mioC* gene (Rokeach *et al.* 1987), the effect of rifampicin may be to disrupt this pausing and thus cause readthrough of *oriC* such that 3' RNA ends are not available for use as primers. If this is indeed the first stage in initiation, with RNA polymerase transcribing *mioC*, the role of DnaA may be to allow such transcripts to be used as primers. DnaA protein may complex with RNA polymerase, protecting the transcripts from degradation, or possibly its role may be to displace RNA polymerase, making these transcripts available for use as primers. Other initiation components such as DnaB and DnaC would then interact in the initiation process. This model assumes that RNA polymerase is responsible for laying down the primer transcript; furthermore, it assumes that the primer transcript has already been synthesised by the time DnaA protein and the rest of the initiation components act.

Models of initiation by Bramhill and Kornberg (1988) and McMacken *et al.* (1987) generally assume that initiation components DnaA, DnaB and DnaC act before the actual priming event; complexing and interacting with *oriC* in such a manner as to allow priming to occur. This is despite the fact that an RNA polymerase step is known

to preceed binding of the DnaA protein at *oriC* (Kaguni and Kornberg 1984). Since both DnaG primase and RNA polymerase are able to act as the priming enzyme in an *in vitro* system (Ogawa *et al.* 1985), it is difficult to know what is a true representation of the real *in vivo* situation. For example, the order of events *in vivo* may be transcription of leading strand primers by RNA polymerase, followed by a DnaA-DnaB-DnaC protein interaction, followed by lagging strand priming by DnaG primase. In an *in vitro* system, the order of events could be the DnaA-DnaB-DnaC interaction, lagging strand priming by DnaG primase, and then leading strand priming (by DnaG primase for example in the *in vitro* system lacking RNA polymerase). Thus the order of events would have been changed, but the end result (i.e. initiation of DNA replication) would be the same. This could presumably only occur in the *in vitro* replication system, where RNA polymerase could be dispensed with. In the following description of the observed stages in the initiation process, it should be emphasised that these represent *in vitro* experimental data, and the precise relationship to the order of events *in vivo* (particularly with regard to RNA polymerase) may not be wholly accurate.

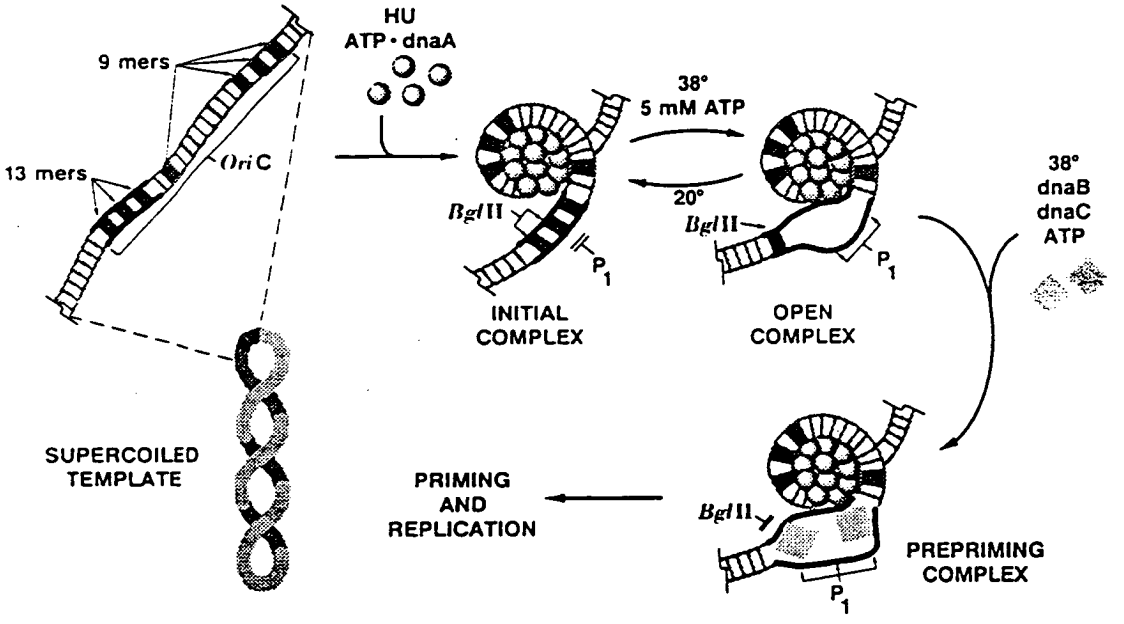
1.4.2 Binding of DnaA to *oriC* and the formation of an open complex

In the *in vitro oriC* replication system, the first stage so far observed involves the binding of 20-40 monomers of DnaA protein to *oriC* (Fuller *et al.* 1984). All forms of DnaA protein are able to form this initial complex (whether DnaA, DnaA-ATP or DnaA-ADP; Sekimizu *et al.* 1988), although only the ATP form is active in forming the subsequent open complex (Sekimizu *et al.* 1987). Because of this, the association of DnaA with ATP may precede the actual binding, and might thus be considered a 'pre-initiation' step. This could therefore provide additional control over the initiation process. A further hypothesis might be that DnaA-ATP is not normally a limiting factor in initiation, but that *oriC* is occluded by some putative protein complex until such time as initiation occurs. This might be removed (as a result of transcription by RNA polymerase for example) to allow the onset of initiation. Such a putative 'occlusion complex' is absent from the *in vitro* replication system, and might explain why initiation can occur continuously. Such a hypothesis might also explain why over-expression of *dnaA* causes over-initiation; large amounts of DnaA being able to displace this putative 'occlusion' complex.

Following the initial binding of DnaA to *oriC*, the initial complex is transformed into an open complex, recognisable as such by its sensitivity to P1 nuclease (Sekimizu *et al.* 1987). Formation of this open complex requires incubation at temperatures

exceeding 30°C, and moreover, once formed it is cold sensitive and must be maintained above 30°C for replicative activity to be maintained (van der Ende *et al.* 1985, Kornberg *et al.* 1987). This is evidently an artifact of the *in vitro* replication system, since *E.coli* is able to replicate its DNA *in vivo* at temperatures less than 30°C. It may be due to the absence from the *in vitro* system of proteins normally involved in separating the strands of duplex DNA, the result being that higher temperatures are required for efficient duplex melting. The potential for suppression can be seen here, either in facilitating efficient formation of the initial multimeric DnaA-*oriC* complex, or in the subsequent formation of the open complex form, possibly by involvement in the duplex melting step.

FIGURE 1.4 A scheme for *oriC* initiation



The DNA in the origin region is proposed to be wrapped round a DnaA protein complex, thus bringing the 13bp DnaB-DnaC 'boxes' alongside and thus allowing duplex opening and subsequent DnaB-DnaC protein entry. Taken from Bramhill and Kornberg 1988.

Formation of the open complex appears to occur at a region comprising three direct repeats of 13 bases in length, indicated in figure 1.3 as the DnaB-DnaC protein complex entry site. P1 nuclease sensitivity studies, together with deletion analysis have identified this region as the site for initial duplex opening by the DnaA protein (Bramhill and Kornberg 1988). Since this region is adjacent to the DnaA boxes to which the protein initially binds, it has been suggested that the DNA is wrapped around the DnaA protein complex to bring these 13-mers alongside, ready for interaction with DnaA and subsequent duplex opening (Bramhill and Kornberg 1988, McMacken *et al.* 1987). This is illustrated diagramatically in figure 1.4.

1.4.3 DnaB, DnaC, and the formation of a prepriming complex

Following the formation of the open complex, the DnaB helicase is delivered in complex with DnaC protein to form a prepriming complex (Wickner and Hurwitz 1975, Kobori and Kornberg 1982a). Its formation depends upon the template DNA being in a negatively supercoiled state, and requires that the incubation temperature exceeds 30°C (van der Ende *et al.* 1985, Kornberg *et al.* 1987), although again, this latter requirement is probably artifactual rather than a representation of the situation *in vivo*. Formation of this prepriming complex is stimulated approximately fourfold upon inclusion of a low level of the *E.coli* histone-like DNA binding protein HU in the reaction mixture (Baker et al 1987), although the mechanistic reason for this is not clear. The exact structure of this prepriming complex is not known, although it is probably comprised of DnaA, DnaB, DnaC and HU proteins, thus providing ample scope for a variety of protein-protein interactions (and consequently interactive suppression).

The next stage in the *in vitro* initiation system appears to be the unwinding of the duplex DNA in the vicinity of *oriC* by the action of DnaB helicase (Baker *et al.* 1986, Kornberg *et al.* 1987). This can be observed as a change in the pattern of P1 nuclease sensitivity over that observed for the DnaA-*oriC* open complex described above (Bramhill and Kornberg 1988). In addition, electron microscopy studies have indicated that an additional 50bp of *oriC* DNA is included in this prepriming complex over that involved in the open complex (Funnell *et al.* 1987). The unwinding action of DnaB helicase depends upon the hydrolysis of rNTPs, and in contrast to the previous steps does not appear to require incubation of the reaction mixture at an elevated temperature (Baker *et al.* 1986). SSB binds to the separated DNA strands at this stage, thus blocking reassociation of the complementary chains. As a result, the prepriming complex is converted to a small bubble-form, which can sustain binding and a limited amount of replication (Sekimizu *et al.* 1988). With the addition of DNA gyrase, which

can provide the necessary swivelling action (but still uncoupled from priming and DNA replication), this small bubble-form enlarges considerably to produce a large unwound structure, known as Form I (Sekimizu *et al.* 1988, Baker *et al.* 1986, Baker *et al.* 1987). It is unclear whether DnaA protein is still bound to *oriC* at this stage; similarly unclear is whether the small bubble-form is responsible for directing the priming reaction, or whether this is the preserve of Form I.

RNA priming is thought to occur once efficient duplex opening has been achieved. Priming *in vitro* can be brought about by any one of three enzyme systems: DnaG primase alone, RNA polymerase alone, or both combined (Ogawa *et al.* 1985). Protein requirements for *in vitro* priming comprise DnaA, DnaB, DnaC, HU protein, SSB, GyrA, GyrB, Topoisomerase I and RNAaseH (to maintain template specificity; van der Ende *et al.* 1985, Ogawa *et al.* 1985, McMacken *et al.* 1987). It is unclear however whether the full repertoire of these proteins is actually required for the priming reaction as such, or whether their requirement is limited to the pre-priming stages. Thus for example, if RNA polymerase is responsible for synthesising the primer transcript, and if DnaA protein is still be in complex with *oriC* at this stage, a route is provided by which suppression of *dnaAts* by secondary mutations in *rpoB* might occur (section 1.5.5). Alternatively, if DnaA is no longer associated with *oriC*, this would suggest that suppression by *rpoB* is due to an interaction well before the actual priming event.

Following priming of the *oriC* template, DNA replication is able to proceed by extension of the primer transcripts by DNA polymerase III. This stage in DNA replication has been covered in section 1.1.3. It is probably clear to the reader that initiation involves a multitude of different proteins and stages, and given the complexity of the process, allows ample scope for the possibility of interactive suppression. With the background to the biochemistry of initiation having now been given, a description of those suppressors of *dnaA* already identified will be presented. This will hopefully allow an appreciation of the mechanisms by which suppression of *dnaA* mutations might occur, particularly with regard to the object of study of this thesis, that of suppression by over-expression of the *groE* genes.

1.5 Extragenic Suppressors Of *dnaA*

1.5.1 Introduction

dnaAts mutants exhibit a remarkably high rate of reversion to temperature resistance, of which only a small fraction is due to true reversion (Atlung 1981), the rest being due to extragenic suppression (Atlung 1981, Wechsler and Zdzienicka 1975). In addition, instances of intragenic suppression occur also, a notable example being the *dnaAcos* mutant mentioned earlier (Kellenberger-Gujer *et al.* 1978). At least 7 loci can be identified for these extragenic suppressor mutations (Atlung 1981). In addition, attempts to clone the *dnaA* gene have identified at least 4 cloned suppressors which can presumably act as such due to their new molecular environment (Projan and Wechsler 1981; Takeda and Hirota 1982; Jenkins *et al.* 1986; Fayet *et al.* 1986), and a variety of partial high-copy suppressors isolated during this work are described in chapter 5. Over-expression due to high copy number would appear to be the most obvious explanation for this behaviour, although Rowen *et al.* (1982), have reported a suppressor of *dnaA* cloned in lambda which appears to function in low copy. However, this is not to say that the cloning of this gene will not have affected the mechanisms involved in controlling its expression. This plethora of suppressors would tend to argue that the DnaA protein participates in a variety of complex cellular processes in interaction with a large number of different gene products.

There are at least 4 general mechanisms by which suppression may occur:

1. Bypass suppression; in which the requirement for a particular gene product is completely eliminated. In the case of DnaA this involves initiation of replication from an origin other than *oriC*, either as a result of a mutation in the host function RNAaseH allowing the use of secondary *E.coli* origins (see below), or by means of the integration of a replicon -which is either independent of, or less dependent upon DnaA function- into the *E.coli* chromosome. This is known as integrative suppression, and a number of examples exist (Nishimura *et al.* 1971; Lindahl *et al.* 1971; Moody and Runge 1972; Nishimura *et al.* 1973; Goebel 1974; Tresguerres *et al.* 1975; Chesney and Rothman-Scott 1978; Molin and Nordstrom 1980).

2. Informational suppressors. Apart from the classic nonsense suppressing tRNAs, frameshift (Smith 1979) and missense (Hill 1975) suppressors exist. It is a characteristic of these suppressors that they exhibit allele specificity independent of the location of the mutation within the gene, suppression depending on the *type* rather than location of that mutation. In addition, they exhibit suppression

of a wide range of otherwise unconnected genes. As with bypass suppression, informational suppression is not particularly informative as to possible protein-protein interactions, nor is it helpful in determining the functional domains within a protein.

3. Increased levels of the mutated polypeptide. This could be achieved by a variety of routes, with an increased amount presumably compensating for a lower specific activity of the protein. Possible routes to increased protein levels might be an increase in the level of transcription, increased translational efficiency, increased stability of the resultant peptide, or better protein processing (if this is required for functional activity). Additionally, higher levels of an active form of the protein might be achieved (for example, in the case of DnaA, the DnaA-ATP complex is active, while the DnaA-ADP form is not; Sekimizu *et al.* 1988). With regard to *dnaA*, there have been reports (quoted in Atlung 1984) that increased expression of certain *dnaAts* alleles is able to overcome the temperature sensitive phenotype and allow growth at the normally restrictive temperature, although whether this is actually a result of increased DnaAts protein levels (since the *dnaA* gene is autoregulatory and therefore increased expression should occur automatically following thermal denaturation; Atlung *et al.* 1985a, Braun *et al.* 1985) remains to be seen.

4. Interactive Suppression. Of the four, this is by far the most illuminating, both in terms of apportioning activity to a particular part of a protein, and for suggesting possible interactions between different gene products. Interactive suppression alone might well be expected to exhibit a degree of allele specificity concomitant with the position of the mutation within the gene.

Regarding suppression of *dnaAts*, although little work has been conducted on determining the mechanism of suppression in most instances, the absolute requirement for DnaA protein for *oriC* initiation (Kaguni and Kornberg 1984) suggests that suppression probably proceeds by routes two, three and four.

1.5.2 Suppression by mutants *dasA, dasB, dasC, dasF and dasG*

None of the above suppressors (Atlung 1981) has been mapped precisely on the *E.coli* chromosome apart from *dasF* (otherwise known as *sin* or *sdrA*), which has in fact been shown to be allelic to *rnh* (RNAaseH, see below). The *dasA* mutation maps at 80 minutes on the *E.coli* genetic map, and results in a cold sensitive phenotype with an increased DNA/protein ratio. As such, the suppression may be

similar in mechanism to the aforementioned intragenic *dnaAcos* suppressor of Kellenberger-Gujer *et al.* (1978), resulting in increased DnaA protein levels or activity. *dasB* maps close to the *gid* gene near *oriC* (Atlung 1981, von Meyenburg and Hansen 1980), and results in a rich medium sensitive phenotype. The mechanism of suppression could therefore be similar to that of RNAseH suppressors of *dnaA* (also rich medium sensitive), in which initiation occurs from origins other than *oriC* (Kogoma and von Meyenberg 1983). *dasC* maps close to *ilv*, and is therefore possibly allelic with the *rep* gene (coding for one of the DNA helicases; Denhardt *et al.* 1972). If so, the function of its gene product, together with the allele specificity which has been reported for this suppressor (Atlung 1981) would argue strongly that some kind of interactive suppression is taking place. *dasG* maps close to the *dnaC-dnaT* cluster and is possibly an allele of one of these genes; since DnaC is intimately involved with DnaA in early stages of the initiation process (see McMacken *et al.* 1987 and Kornberg *et al.* 1987), and *dnaT* encodes one of the primosomal proteins (protein i; Masai *et al.* 1986), suppression by an allele of one or other of these genes would seem plausible. *dasE* maps at approximately 1 minute on the genetic map. Since it was originally isolated in conjunction with the *dasG* allele, it is unclear whether it is a suppressor of *dnaAts* mutations in its own right, or whether it is merely a secondary mutation allowing more efficient suppression by *dasG*.

1.5.3 Suppression by *topA*

A Mutation in the *topA* gene of *E.coli*, which codes for topoisomerase 1 is able to suppress the *dnaA46ts* mutant (Louarn *et al.* 1984). Suppression requires the presence of the DnaA protein, and the authors suggest it may be caused either by over-production of the mutant DnaA46 protein due to a change in the superhelical density of the DNA (Pruss *et al.* 1982; Dinardo *et al.* 1982), or, that initiation at *oriC* requires less DnaA protein in the absence of topoisomerase 1.

1.5.4 Suppression by RNAaseH mutants

Recent work by several groups (Horiuchi *et al.* 1984; Lindahl and Lindahl 1984; Ogawa *et al.* 1984) has demonstrated that mutant strains of *E.coli* deficient in RNaseH are able to suppress *dnaAts* mutations. It has become apparent that the four loci *dasF*, *sdrA*, *rin* and *rnh* are all in fact alleles of the same gene, that encoding RNAaseH (Lindahl and Lindahl 1984, Torrey *et al.* 1984). All *dnaA* alleles can be suppressed by mutations in RNAaseH, including those that have been insertionally inactivated, and the fact that *oriC* can be deleted from these strains indicates that in some instances at least, suppression is bypass in nature (Kogoma and von Meyenburg

1983). That is, initiation is occurring from origins other than *oriC*, and the resultant changes in gene dosage compared to a normal strain during growth are probably the cause of the rich medium-sensitive phenotype of these mutants (Kogoma and von Meyenburg 1983). Normally RNaseH destroys the RNA moiety of RNA:DNA hybrids, and is important in maintaining the specificity of initiation form *oriC* In the absence of this enzyme, RNA:DNA hybrids formed at secondary replication origins are not destroyed, and are therefore presumably able to direct initiation (de Massy *et al.* 1984). However, since RNAaseH and DnaA are both constituents of the *in vitro* replication system, the possiblity that both proteins may interact at *oriC* (DnaA may protect primer transcripts from excessive processing by RNAaseH for example), provides ample scope for interactive suppression also (Ogawa *et al.* 1985).

1.5.5 Suppression by mutations in *rpoB*

Suppression of *dnaA* mutants by secondary mutations in the *rpoB* gene are possibly the most interesting type, if only because they are the most intensively studied and therefore most informative (Atlung 1981; Atlung 1984; Schaus *et al.* 1981a; Hansen *et al.* 1984). All *dnaAts* alleles can be suppressed by secondary *rpoB* mutants; however they display allele specificity as to which of the *dnaAts* mutations they suppress (Atlung 1984). In addition, one of the *dnaA* amber mutations is suppressed by *rpoB* (Schaus *et al.* 1981a), leading on to the possibility that suppression is informational or bypass in nature. The latter possibility seems unlikely though, since other *dnaAam* mutants tested were not suppressed, suggesting that at least a partially functional *DnaA* protein is required for suppression to occur. This is further borne out by the position of the suppressed amber mutation within the gene; mapping to its distal end, and as such, leaving most of the protein intact (Schaus *et al.* 1981b).

A comparison of the map position of various temperature sensitive alleles of *dnaA* with the pattern of suppression by *rpoB* shows some highly interesting correlations (figure 1.5). All the *dnaA* mutations located in the central portion of the gene are suppressed by four *rpoB* alleles; these alleles being unable to suppress the mutations at either end of the gene. These peripheral mutants are suppressed, but a different *rpoB* allele is required for each end. In addition, the *rpoB* alleles which suppress these end mutations are themselves unable to suppress those located in the central portion of the gene. This would seem to suggest that the mechanism of suppression of mutations located in the central and peripheral portions of the *dnaA* gene is different. Suppression by secondary mutations in *rpoB* is thus a strong candidate for interactive suppression, especially since DnaA and RNA polymerase are

both known to act at an early stage in initiation (Fuller and Kornberg 1983). In addition, these results would suggest at least two, and possibly three, functional domains for the DnaA protein. However, it should be remembered that a two dimensional linear map of allele location within a gene need not necessarily bear much resemblance to the location within the three-dimensional protein structure. Regardless of this, the correlation still appears striking.

FIGURE 1.5 Allele specificity of suppression of *dnaAts* mutations by secondary mutations in *rpoB*

	dnaAts allele no. (location with respect to promoter of *dnaA*)									
	← *dnaA*									P
	203	*204*	*602*	*601*	*604*	*606*	*5*	*46*	*167*	*508*
rpoB902	−	−	+	+	+	+	+	+	+	−
rpoB903	+	+	−	−	−	−	−	−	−	−
rpoB904	−	−	−	−	−	−	−	−	−	+

Data taken from Atlung 1984 and Hansen et al. 1984

Apart from a direct protein-interaction mediated suppression at *oriC*, it is possible that suppression results from altered promoter specificities of the mutant RNA polymerase, leading to over-expression of a partially functional DnaA protein. There have been reports (quoted in Atlung 1984) that over-expression of certain mutant *dnaA* alleles leads to suppression of the temperature sensitive phenotype. However, the pattern of allele specificity differs from that of the *rpoB* suppressors, suggesting that over-expression is not the mechanism for *rpoB* suppression. In addition, altered promoter specificities should lead to multiple phenotypic effects on the cell; these have not been reported although it must be said that a detailed study of possible side effects of *rpoB* suppression has not been conducted. Finally, since the *dnaA* gene is autoregulatory, it would seem likely that increased expression would occur automatically following thermal denaturation of the DnaAts protein (Atlung *et al.* 1985b, Braun *et al.* 1985).

It is possible that suppression might proceed by a different mechanism, dependent upon the *rpoB* allele involved, such that interactive suppression occurs with

certain alleles (e.g. those located in the central portion of the *dnaA* gene), with over-expression being the mechanism by which other alleles are suppressed (e.g. those located at one of the ends). Finally, the fact that a large proportion of *rpoB* mutant alleles result in increased supercoiling of the DNA (R. Menzel, pers. comm. to McMacken *et al.* 1987) suggests that suppression may be mediated indirectly (for example increased supercoiling may aid the mutant DnaA protein in forming an initiation complex at *oriC* in an analogous manner to that suggested for suppression by topoisomerase I). However, given the data on allele specificity, plus the knowledge that both DnaA and RNA polymerase act both spatially and temporally closely together in the initiation process, would argue strongly for the likelihood of some form of interactive suppression.

1.6 The Project – The Mechanism Of Suppression By The *groE* Genes When Cloned In High Copy Number

1.6.1 Cloned suppressors

At least 5 separate cloned suppressors of *dnaA* have been isolated (Projan and Wechsler 1981; Takeda and Hirota 1982; Jenkins *et al.* 1986; Fayet *et al.* 1986), and results presented in chapter 5 of this thesis describing the isolation of a variety of partial *dnaA* suppressors suggest that this may not be the full complement of cloned suppressors available. Of these five, only one has been characterised, the product of the genes *groEL* and *groES* (Jenkins *et al.* 1986; Fayet *et al.* 1986). Little work has been done on assigning chromosomal loci to the other suppressors, although their restriction maps and complementation analysis have enabled the elimination of certain genes as being involved, notably *dnaB, dnaC, dnaE, dnaZ* and *rpoB*. They are unable to suppress amber mutations, suggesting that suppression is not bypass in nature. Apart from this limited information, nothing else is known about either the nature of the suppression, nor of the genes involved.

The GroE proteins (GroES and GroEL) are amongst the most abundant polypeptides in the *E.coli* cell, and yet despite this, little is known about their normal cellular role. They have been implicated in bacteriophage head assembly, they are known to be heat shock proteins, when over-produced or mutated they can suppress temperature sensitive mutations in DNA replication proteins (notably DnaA and SSB; Fayet *et al.* 1986, Jenkins *et al.* 1986, this thesis, Ruben *et al.* 1988) and they have been implicated in coupling F plasmid replication to host cell division (Miki *et al.* 1988). They have been shown to interact with ribosomes (Neidhardt *et al.* 1981), and an interaction with RNA polymerase is suggested by the finding that an altered subunit of

RNA polymerase is able to suppress a mutation in *groES* (Wada *et al.* 1987). In spite of this, the only known enzymatic function of either of these proteins is a weak ATPase activity for GroEL (Ishihama *et al.* 1976, Hendrix 1979). Can this plethora of interactions and functions give some clue about the normal role of the GroE proteins in the cell, and particularly with regard to how suppression of *dnaAts* mutations might occur?

The *groE* gene products are heat shock proteins; that is, they are preferentially expressed following exposure of the cell to a variety of stress inducing agents (of which 'heat shock' itself is only one example; Neidhardt *et al.* 1984, Neidhardt and VanBogelen 1987). For this reason their role may be thought of as being to somehow make the cell more 'robust' to such changes, and the mechanisms by which this is brought about may have implications regarding the functions of both GroES and GroEL. For the reader to fully appreciate the available data concerning these proteins (described in the following section), a brief description of the current knowledge of the *E.coli* heat shock response shall first be presented.

1.6.2 The *E.coli* heat shock response

The heat shock response is a seemingly universal phenomenon which can be seen across the eukaryotic and prokaryotic kingdoms following a shift up in temperature, with many of the individual constituent proteins being highly conserved across evolutionary time (for reviews see Schlesinger *et al.* 1982, Neidhardt and VanBogelen 1987, Pardue 1988). In addition to temperature, agents that induce the heat shock response in *E.coli* include ethanol, puromycin, viral infection, nalidixic acid, hydrogen peroxide and amino acid starvation (Neidhardt and Van Bogelen 1987). In general, although it is not known precisely why this selection of agents should induce the heat shock response, a reasonably common element would appear to be the accumulation of damaged proteins, or else a gross disruption of normal cell physiology (for example the cessation of DNA replication).

Induction of the heat shock response is under the control of the *htpR* gene, a positively acting regulatory gene which encodes a sigma-like transcription factor specific for heat shock genes (including its own promoter; it is therefore a positive regulator of its own synthesis; Neidhardt and VanBogelen 1981, Grossman *et al.* 1984, Grossman *et al.* 1987). For this reason the *htpR* gene is also referred to as *rpoH* and its protein product as σ^{32} (Grossman *et al.* 1985). The heat shock regulon comprises a complement of 17 proteins so far identified, although further proteins may await discovery (Herendeen *et al.* 1979, Neidhardt and Van Bogelen 1987). Apart from GroES

and GroEL, other heat shock proteins include DnaK, DnaJ, Lon, HtpG, GrpE, RpoD, HtpR, LysU and HtpE, H, I, K, L, M, N, O (with the latter set being as yet identified only as spots on 2D gels; Neidhardt *et al.* 1983). The precise role of many of these proteins in the heat shock response is unclear, although some of them seem more important than others (Kusukawa and Yura 1988). HtpR (σ^{32}) has already been mentioned as a regulator of the heat shock response; DnaK appears to be important in this respect also. DnaK is a protein kinase of an extremely conserved class (the *Drosophila* analogue shows 50% homology), and appears to function by phosphorylating, and thus inactivating σ^{32} (Zylicz *et al.* 1983, Bardwell and Craig 1984, Tilly *et al.* 1983). It is thus a negative regulator of the heat shock response. Also acting in a negative regulatory capacity is the *rpoD* gene product (encoding σ^{70}), a sigma factor which is only able to recognise normal *E.coli* promoters in contrast to σ^{32} which recognises heat shock promoters (Neidhardt and VanBogelen 1987). Therefore it seems likely that the two sigma factors, σ^{32} and σ^{70} are in direct competition with each other and therefore serve to modulate the level of the heat shock response (Grossman *et al.* 1985). Until recently there has been uncertainty over whether the normal low level expression of heat shock genes in the absence of induction was due to inefficient expression by normal physiological levels of RNA polymerase plus σ^{70}, or whether efficient expression from low levels of RNA polymerase plus σ^{32} was occurring (Grossman *et al.* 1984, Bloom *et al.* 1986). The recent discovery that *htpR* null mutants are non-viable at temperatures above 20°C (Zhou *et al.* 1988) suggests that the latter is probably the true situation, with low levels of σ^{32} normally present in the cell.

What is the function of the heat shock response, and why should this particular range of proteins be induced? At least part of the function appears to be the degradation of abnormal proteins, presumably the result of heat-denaturation. One of the above mentioned genes, *lon*, encodes the La protease, the only *E.coli* protease involved in the degradation of abnormal proteins which has been identified genetically (Charette *et al.* 1981, Chung and Goldberg 1981). *lon* mutants exhibit a reduced rate of proteolysis of abnormal proteins, although since the rate of preoteolysis is reduced still further in a *htpR* mutant strain, this would suggest that additional (as yet unidentified) proteases transcribed using σ^{32} play a significant role in abnormal protein degradation (Goff *et al.* 1984). The rate of abnormal protein degradation can be both elevated and reduced in a *dnaK* mutant strain, agreeing with the premise that this gene product may be involved in a regulatory role in the heat shock response (Keller and Simon 1988). Further evidence linking the heat shock response to proteolysis can be seen in the induction of the heat shock response following the production of

abnormal foreign proteins (Goff and Goldberg 1985), as well as by amino acid analogues which result in abnormal *E.coli* proteins.

However, proteolysis would seem an unlikely route by which suppression of *dnaAts* by *groE* might occur. Induction of proteases would seem more likely to result in the degradation of a thermally denatured DnaAts protein. However, as well as resulting in the proteolysis of damaged proteins, the heat shock response is likely to be concerned with protecting cells from the effects of stress-inducing agents in other ways. In this respect, recent work by Kusukawa and Yura (1988) is highly revealing. As has previously been mentioned, null mutants of σ^{32} are unable to grow at temperatures above 20°C, indicating a low level requirement for heat shock proteins under normal growth conditions. However, if the GroE proteins are supplied from a promoter independent of σ^{32}, then growth of the same strains becomes possible up to 40°C, indicating that the GroE proteins play a key role in allowing growth at higher temperatures. The exact roles of the *groE* gene products is unclear, although since they appeared to be involved in macromolecular assembly (see the following section and chapter 3), they probably fulfil some sort of structural role in the heat shock response, possibly stabilising partially denatured proteins or structures. Since other constituents of the heat shock response are not induced under these conditions (and yet the cell is viable up to 40°C), this would suggest that other heat shock proteins are of subsidiary importance. Furthermore, enhanced synthesis of DnaK (in addition to GroESL) enables growth up to 42°C in the complete absence of other heat shock proteins. Since neither DnaK or GroESL are proteases, this would suggest that the primary function of the heat shock response is not proteolytic, but instead is probably concerned with stabilising cellular components, possibly protecting proteins from degradation (this would agree with the finding that certain *dnaK* mutants exhibited increased proteolysis of abnormal proteins (Keller and Simon 1988), and in this respect it would perhaps be interesting to discover if the same holds true for *groE* mutant strains).

1.6.3 The *groE* genes

The *E.coli groE* genes were first identified as mutations affecting the assembly of the heads of bacteriophages T4 and λ (Takano and Kakefunda 1972, Georgopoulos *et al.* 1973, Sternberg 1973, Coppo *et al.* 1973), with subsequent work revealing the involvement of the GroE proteins in phage T5 tail assembly (Zweig and Cummings 1973). For this reason the *groE* genes are also known as *mop* (morphogenesis of phage), with *groES* being known as *mopB* and *groEL* as *mopA* (Bachmann 1983). The

groE locus maps to 94 minutes on the genetic map (Georgopoulos and Eisen 1974), and is comprised of two genes, *groES* and *groEL* which are cotranscribed as a single unit from a promoter upsteam of *groES* (Tilly *et al.* 1981, Hemmingsen *et al.* 1988). The *groE* genes may therefore be thought of as comprising an operon, and the promoter controlling transcription is recognised by the heat shock sigma factor σ^{32} (Cowing *et al.* 1986).

As previously mentioned, the *groE* genes form part of the *E.coli* heat shock regulon, although the genes are essential for growth under normal conditions, since null mutants are inviable (quoted in Hemmingsen *et al.* 1988). In addition, a variety of temperature sensitive mutations in both genes have been identified (Georgopoulos and Eisen 1974, Tilly *et al.* 1981, Wada and Itikawa 1984). On the basis of these results, the GroE proteins are evidently required during normal cellular growth, although no role for either proteins has yet been ascertained. DNA sequence analysis has given molecular weights for these proteins of 10.368 and 57.259 kd for GroES and GroEL respectively (Hemmingsen *et al.* 1988), in reasonably good agreement with apparent relative masses of 15.000 and 65.000 kd (Tilly *et al.* 1981, Georgopoulos and Hohn 1978). Synthesis of the GroE proteins appears to be related to the cellular growth rate, with more protein being produced as the growth rate increases (Pedersen *et al.* 1978). Even in the absence of heat shock induction they are amongst the most abundant proteins in the cell; GroEL makes up 0.8–1.2% of cellular protein under normal growth conditions, rising to 12% at 42°C (Pedersen *et al.* 1978). This high level of induction would tend to suggest that the GroE proteins are among the more important of the heat shock proteins, this finding being borne out by the observation that induction of GroESL alone is sufficient to allow cellular growth up to 40°C.

Both GroE proteins are found in the cell as oligomeric structures, with GroEL being present as a 14 subunit homo-oligomer composed of two stacked rings of 7 subunits each (Hendrix 1979, Hohn *et al.* 1979). This finding that the GroEL multimeric form has 7-fold rotational symmetry may be significant regarding an involvement in phage head assembly. Proteins with 7-fold symmetry are rare in nature; indeed, the GroEL complex is the only such one identified in *E.coli* so far. The fact that both the λ and T4 phage head assemblies have triangulation numbers of 21 suggests a possible structural role for GroEL in their assembly. The GroES protein is found as a single ring of 6–8 identical subunits in the cell (Chandrasekhar *et al.* 1986). On the basis of intergenic suppression and the fact that mutations in either gene are phenotypically indistinguishable, it has been proposed that the two proteins interact *in vivo* (Tilly and Georgopoulos 1982). *In vitro* evidence seems to bear this out. GroEL is a weak

ATPase, and is able to form a complex with GroES in the presence of Mg-ATP but not in its absence (Chandrasekhar *et al.* 1986).

The products of both genes are required for the morphogenesis of phages λ and T5, although only GroEL appears to be necessary for T4 morphogenisis (Tilly and Georgopoulos 1982). The GroE proteins appear to act at an early stage in the head assembly pathways; in the λ head assembly pathway, both GroE proteins are required for the earliest stage so far detected, the formation of an oligomer of 12 subunits of phage protein gpB (Kochan and Murialdo 1983). This interaction is perhaps particularly interesting in view of the amino acid homology which is present between GroEL and gpB, possibly suggesting a similar function for this part of the proteins (discussed in chapter 3). Phage T4 requires GroE for cleavage of a tail protein during assembly (Zweig and Cummings 1973), and an interaction between GroEL, gp23 (the major T4 head protein) and gp31 in T4 head assembly seems likely on the basis of interactive suppression (Revel *et al.* 1980, Takahashi *et al.* 1975). In the absence of a functional GroEL, T4 gp23 is found associated with the cell membrane in amorphous clusters, in agreement with the premise that GroEL is involved in assembly of the phage head structure (Takano and Kakefunda 1972).

This role of the GroE proteins in the assembly of macromolecular structures, together with the fact that they are not found as part of the assembled head structure (Friedman *et al.* 1984) has led to the classification of GroE as a 'molecular chaperone' (Hemmingsen *et al.* 1988). That is, a protein involved in post-translational assembly of oligomeric protein structures. This concept is discussed more fully in chapter 3, but in the case of GroEL such a role is suggested by extensive amino-acid homologies to a protein of similar function found in the chloroplasts of higher plants, called the Rubsisco subunit binding protein (Hemmingsen *et al.* 1988). This conservation of amino acid sequence across evolutionary time is remarkable, and would tend to argue for the presence of severe evolutionary constraints upon the GroE protein type. In addition to the aforementioned homology, GroEL shows homology to a similar 7-subunit protein of *Bacillus subtilis* (Carrascosa *et al.* 1982), to a nuclear-encoded mitochondrial heat shock protein of several eukaryotic species (McMullin and Hallberg 1988), and to proteins from a variety of different bacterial species including archaebacteria (Young *et al.* 1987, quoted in Hemmingsen *et al.* 1988).

The homology between GroEL and the *Mycobacterial* protein is particularly interesting, since this protein appears to be the major immunogen in a variety of bacterial infections. Indeed, a notable feature of this protein type appears to be their

remarkable antigenicity (Young *et al.* 1988). This is perhaps all the more suprising considering the high level of conservation of the GroEL protein type; ordinarily proteins which display little evolutionary difference between species illict a poor immunogenic response (Lerner 1982). A possible explanation for the strength of the immunogenic response against the GroEL-type antigen has been suggested by Young (quoted in Pardue 1988). He has suggested that the immunogenicity might be due both to a strong induction of heat shock in the pathogen when it invades the host and to the conserved structure of heat shock proteins. As a result of this conservation, the immune system of the host has a good probability of a previous encounter with epitopes similar enough to ellict a strong response. This may be true; however, it fails to explain why antigenically-related proteins in the host cells' mitochondria should not continually be subject to auto-immunogenic recognition.

1.6.4 Objectives of the project

From the above, it can be seen that the GroE proteins have at least two identifiable cellular functions, although the mechanistic involvement of the proteins in each is perhaps less clear. These are (1) involvement in the host cell heat shock response (although both gene products are required for growth under normal conditions also), and (2) an involvement in phage morphogenesis. Both of these phenomena suggest mechanisms by which suppression of *dnaAts* mutations may occur. The function of GroE as heat shock proteins may suggest a role in 'repairing' heat denatured proteins, or protecting them from degradation by cellular proteases. Thus GroE may serve to protect a mutant DnaAts protein from degradation. The involvement of the GroE proteins in phage head assembly suggests a structural role, possibly in the assembly of complex macromolecular structures. Thus GroE might act to stabilise multiprotein complexes present at the origin of replication in which the mutant DnaA protein might normally be unable to act.

However, in addition to the above, a wider range of possibilities becomes apparent if one considers the full range of known interactions of the GroE proteins and the possible implications thereof. Whether these represent real cellular interactions, or are merely due to the fact that a protein as common as GroE is likely to be a major contaminant in any purification procedure cannot be ascertained. However, they have important implications concerning the mechanism by which suppression may be occuring. For example, GroE has been reported to be associated with ribosomes (Neidhardt *et al.* 1981); this immediately raises the possibility of informational suppression. Similarly, both genetic (Wada *et al.* 1987) and biochemical data (Hendrix

1979, Ishihama *et al.* 1976) have suggested an association between GroEL and RNA polymerase. Thus suppression may be due to increased transcription of the *dnaA* gene as a result of GroE-mediated affects on RNA polymerase. Alternatively, this may represent an interaction between GroE, DnaA and RNA polymerase at *oriC*.

The experiments described in this thesis were aimed at narrowing down the possible mechanisms by which suppression might be occurring, to discover if informational suppression, interactive suppression, or bypass suppression might be occurring. Additionally, an effect on the level of the DnaA protein was examined, to try to discover whether elevated mutant DnaA protein levels were the mechanism of suppression (either by increased levels of transcription or translation of the *dnaA* gene, or possibly due to increased stability of DnaA in the presence of extra GroE).

In doing so it was hoped to gain an insight into not only the mechanism of suppression, but also the roles of the respective proteins in the cell. While examining this question, a mutation originally isolated as having lost the ability to mediate suppression above 40°C was isolated. This was expected to provide information into the mechanisms of suppression. In fact, it proved to be a new and previously unidentified *E.coli* gene. The mutant allele was found to result in a lower copy number of certain plasmids, and was deemed worthy of study not only because of the novel nature of the mutation (no other host mutants exclusively affecting plasmid replication having been isolated), but also because it was considered likely that the gene may encode one of the three unidentified primosomal proteins. Insights into host cell DNA replication were therefore anticipated. Finally, an investigation into the measurement of quantitative hybridisation as a means of measuring plasmid copy number was undertaken for two reasons. Firstly, because an accurate measurement of copy number was important on a number of occasions during the course of the experiments described in this thesis, with there being no suitable alternatives available, and secondly, because the measurement of copy number was deemed to be an interesting question in its own right.

CHAPTER 2

Materials and methods

2.1 Bacterial and phage strains

Bacterial strains used in this study are listed in table 2.1. Bacteria were maintained on L-broth plates stored at 4°C for regular use, or in 0.7% nutrient agar stabs at room temperature for longer term storage. In addition, unstable strains (strains carrying unstable/deleterious plasmids etc.) were stored at -70°C or -20°C if more frequent access was required. To prepare for storage, fresh overnight cultures of the strain were harvested by centrifugation and resuspended in $^{1}/_{10}$ volume of 50% glycerol: 50% phosphate buffered saline (table 2.4).

Phage strains used in this study are listed in table 2.2. Phage lysates were stored at 4°C as broth suspensions to which a few drops of chloroform had been added to prevent microbial growth.

2.2 Growth media and buffers

Growth media and phage/bacterial buffers are listed in table 2.4. L-broth and L-agar were used for all routine bacterial manipulations except where stated. For work with phage P1 the media was supplemented with 2.5mM $CaCl_2$, and for phage λ with 10mM $MgSO_4$ and 0.2% maltose to maximise expression of the λ receptor protein. VB agar supplemented with appropriate carbon sources, vitamins and amino acids (table 2.5) was used for the selection of nutritional markers.

2.3 Bacterial techniques

2.3.1 Growth of bacteria

Bacteria were routinely grown as liquid cultures at 37°C. Usually fresh overnight cultures which had been innoculated from a single colony were diluted back the following day and grown as required.

2.3.2 Selection of antibiotic resistance

The routine concentrations and storage conditions for the antibiotics used in this work are shown in table 2.6. Higher concentrations of tetracycline, chloramphenicol and ampicillin were sometimes used when attempting to determine plasmid copy number (see chapter 6). All antibiotics were used in both complex and minimal media with the exception of trimethoprim which was used in minimal media only.

TABLE 2.1 Bacterial Strains

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
159	F-, uvrA157, galK2, rpsl200, (Pa Pa+)	N Murray
621	F-, leuB6, lacA, lacY1, thi-1, recD, supE44, fhuA21;T1R,Ph80R, l-	N Murray, recD derivative of C600
AB1157	F-, DEL: gpt-pro;62, argE3, his4, leu-6, thr-1, ara-14, galK2 lacY1, xyl-5, mtl-1, supE44, rpsL31; StrR, tsx-33;T6R, l-	Howard-Flanders et al. 1964
AB259 (HfrH)	Hfr: PO1; pil > pyrB, thi-1, relA, l-	Jacob & Wollman, 1956
AT986	Hfr (PO45 of Hfr KL16), dapD8, relA1, spoT1, thi-, l-	Bukhari & Taylor 1971, CGSC
BMH7118	DEL: lac-pro, F' lacIq lacZDM15 pro+ supE	Messing et al. 1977
C600	F-, leuB6, lacA, lacY1, thi-1, supE44, fhuA21;T1R,Ph80R, l-	Appleyard, 1954
CB831	F-, DEL: (gpt-proA)62, arg-, leu-, ara-14, galK2, lacY1, xyl-5 mtl-1, thi-1, hsdS20(r- m-), recA13, rpsL;StrR, pVH1	M. Bagdasarian
CG236	galE, supo, groEL44	C Georgopoulos
CG25	galE, supo	C Georgopoulos
CG714	galE, supo, groEL140	C Georgopoulos
CM1843	F-, DEL (atpB-gidA-oriC-asnA)1071, asnB32, ilv192, lysA, fuc-, thi-1 relA1, spoT1::pKN500; KanR, lmb-	von Meyenburg and Hansen 1980
CM2733	F-, his-4, metE46, trp-3, ara-9, galK2, lacY1/Z4, mtl-1, thi-1 supE44, dnaA602ts, fhuA1;T1R,Ph80R, rbs-, rpsL8/9?;StrR, tsx-3; T6R, l-	Hansen et al. 1984

table 2.1: (continued)

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
CM2735	F-, his-4, metE46, trp-3, ara-9, galK2, lacY1/Z4, mtl-1, thi-1 supE44, dnaA601ts, fhuA1;T1R,Ph80R, rbs-, rpsL8/9?;StrR, tsx-3; T6R, l-	Hansen et al. 1984
CM2738	F-, his-4, metE46, trp-3, ara-9, galK2, lacY1/Z4, mtl-1, thi-1 supE44, dnaA604ts, fhuA1;T1R,Ph80R, rbs-, rpsL8/9?;StrR, tsx-3; T6R, l-	Hansen et al. 1984
CM2740	F-, his-4, metE46, trp-3, ara-9, galK2, lacY1/Z4, mtl-1, thi-1 supE44, dnaA606ts, fhuA1;T1R,Ph80R, rbs-, rpsL8/9?;StrR, tsx-3; T6R, l-	Hansen et al. 1984
CM748	F-, his-4, metE46, trp-3, ara-9, galK2, lacY1/Z4, mtl-1, supE44, dnaA205ts, fhuA1;T1R,Ph80R, rbs-, rpsL8/9?;StrR, tsx-3;T6R, l-	Hansen et al. 1984
DK201	sdrA224; rnh-, asnB50::Tn5, ilv192, dnaA5, DEL (atpB-gidA-oriC-asnA)1071	Kogoma and von Meyenburg 1983
DK249	F-, argH, his29, ilv-, metB1, metD88, thyA, trpA9605, deoB/C, dnaA850::Tn10; TetR, rpoB; RifR, sdrA224	Kogoma and von Meyenburg, 1983
DS410	F-, thi-, sup-, minA, minB, rpsL;StrR	Dougan & Sherrat 1977
E11022	his-, trp-, Dlac, T6R, SmR, SpcR, /F'his (F-57)	Laboratory Stock
ED2433	Hfr, argE >, DEL (gpt-lac)5, thi-, met-	Laboratory stock
EH3791	asnB32, relA1, spoT1, thi-1, fuc-1, lysA, ilv-192, zia::pKN500, dnaA::Tn10; TetR, mad-1	Hansen and Yarmolinsky 1986
EH3827	asnB32, relA1, spoT1, thi-1, fuc-1, lysA, ilv-192, zia::pKN500, DEL dnaA	Hansen and Yarmolinsky 1986
G6	Hfr: P0101; argG > Lys, hisA323, l-	Matney et al. 1964

table 2.1: (continued)

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
H1175	F-, argF58, argI60, leu32, proA35, purA35, gal-6, lacY1, malA1;LmbR, mel-, xyl-7, mtl-2, supE44, fhuA48;T1R, Ph80R, rpsL125;StrR, tsx-70; T6R, l-	Glansdorff et al. 1967
HB101	F-, DEL: (gpt-proA)62, arg-, leu-, ara14, galK2, lacY1, xyl-5, mtl-1 thi-1, hsdS20 (r- m-), recA13, rpsl;StrR	Boyer & Roulland-Dussoix 1969
HX24	groES30	R Hendrix
HX369	groEL44	R Hendrix
HX39	groES619	R Hendrix
JM18	F-, argG6, asnA31, asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, 'pcn'	P1Kc on MM18, transduced JM21 to dnaA+ (tempR), screened for asn-. This work.
JM182-1	F-, argG6, asnA+, asnB32, hisG1, leuB6, metB1, pyrE, gal-6, lacY1, xyl-7, supE44, bgl+, dnaA5ts, fhuA2;T1R,Ph80R, gyrA;NalR, rpsL104;strR srl-, tsx-1;T6R, uhp-,l-	Spontaneous low copy derivative of MM182. This work.
JM182-5	F-, argG6, asnA+, asnB32, hisG1, leuB6, metB1, pyrE, gal-6, lacY1, xyl-7, supE44, bgl+, dnaA5ts, fhuA2;T1R,Ph80R, gyrA;NalR, rpsL104;strR srl-, tsx-1;T6R, uhp-,l-	Spontaneous low copy derivative of MM182. This work.
JM182-8	F-, argG6, asnA+, asnB32, hisG1, leuB6, metB1, pyrE, gal-6, lacY1, xyl-7, supE44, bgl+, dnaA5ts, fhuA2;T1R,Ph80R, gyrA;NalR, rpsL104;strR srl-, tsx-1;T6R, uhp-,l-	Spontaneous low copy derivative of MM182. This work

table 2.1: (continued)

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
JM182-9	F-, argG6, asnA+, asnB32, hisG1, leuB6, metB1, pyrE, gal-6, lacY1, xyl-7, supE44, bgl+, dnaA5ts, fhuA2;T1R,Ph80R, gyrA;NalR, rpsL104;strR srl-, tsx-1;T6R, uhp-,l-	Spontaneous low copy derivative of MM182. This work
JM19	F-, argG6, asnA31, asnB32, Dhis1, leuB6, metB1, pyrE, thr1, folA gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R, Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, 'pcn'	P1Kc on N1270, transduced JM18 to TmpR, screened for thr-. This work.
JM21	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA46ts, 'pcn'	Spontaneous low copy derivative of MM185. This work.
JM600	F-, leuB6, lacA, lacY1, thi-1, supE44, fhuA21;T1R,Ph80R, l-, rpoB;rifR	P1kc on TOE12, transduced C600 to rifR. This work
KH1192	Hfr Cavelli, metD88, metB1, proA3, lac3, tsx76, relA1, rnh::Tn3 AmpR	T Horiuchi
KH1331	Hfr Cavelli, metD88, metB1, proA3, lac3, tsx76, relA1, dnaE293	T Horiuchi
KL16	Hfr: PO45; lysA > serA, thi-1, relA1, l-	Lom 1973
LC906	F-, leuB6, thyA47, deoC3, dnaA5ts, rpsL163;StrR	P Carl; L Caro
LN1349	F-, dnaC28ts, thyA, deoB/C, gyrA, thi, trp::Mu+, zib501::Tn10 (l cI ind)	O Fayet
MM17	F-, argG6, asnA31 , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, l-, tsx-1;T6R, uhp-, recA56	J H Pringle, Laboratory Stock

table 2.1: (continued)

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
MM18	F-, argG6, asnA31 , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-,l-	J H Pringle, Laboratory Stock
MM181	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA167ts	P1Kc on WM1029, cotransduced with asnA into MM18. This work.
MM182	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA5ts	P1Kc on LC906, cotransduced with asnA into MM18. This work.
MM183	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA204ts	P1Kc on WM1152, cotransduced with asnA into MM18. This work.
MM184	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA508ts	P1Kc on WM1032, cotransduced with asnA into MM18. This work.
MM185	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA46ts	P1Kc on WM1026, cotransduced with asnA into MM18. This work.
MM186	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA203ts	P1Kc on CM748, . cotransduced with asnA into MM18. This work.

table 2.1: (continued)

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
MM187	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA602ts	P1Kc on CM2733, cotransduced with asnA into MM18. This work.
MM188	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA601ts	P1Kc on CM2735, cotransduced with asnA into MM18. This work.
MM189	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA604ts	P1Kc on CM2738, cotransduced with asnA into MM18. This work.
MM19	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, ::Tn7;TmpR, tsx-1;T6R, uhp-, l-, dnaA46ts	I R Oliver, Laboratory Stock
MM190	F-, argG6, asnA+ , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, dnaA606ts	P1Kc on CM2740, cotransduced with asnA into MM18. This work.
MM20	F-, argG6, asnA , asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R,Ph80R, uhp-, l-, polAamb	I R Oliver, Laboratory Stock

table 2.1: (continued)

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
MM22	F-, argG6, asnA31, asnB32, Dhis1, leuB6, metB1, pyrE gal-6, lacY1, xyl-7, supE44, bgl+, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, srl-, tsx-1;T6R, uhp-, l-, recA56, dnaA46ts	J H Pringle, Laboratory Stock
MM28-2	F-, argG6, asnA31, asnB32, Dhis1, leuB6, metB1, pyrE, gal-6, lacI-, lacY1, xyl-7, supE44, bgl+, fhuA2; T1R, Ph80R, gyrA; NalR, rpsL104; StrR, srl- tsx-1; T6R, uhp-, 'pcn', recA56	I R Oliver. recA, LacI- version of JM18
MM7	F-, argG6, hisG1, ilv-, leuB6, metB1, pyrE gal-6, lacY1, malA1;LmbR, xyl-7, supE44, fhuA2;T1R,Ph80R, gyrA;nalR rpsL104;StrR, tsx-1;T6R, uhp-, l-	Laboratory Stock
MM9	F-, argG6, hisG1, ilv-, leuB6, metB1, pyrE gal-6, lacY1, malA1;LmbR, xyl-7, supE44, fhuA2;T1R,Ph80R, gyrA;nalR recA56, rpsL104;StrR, tsx-1;T6R, uhp-, l-	Laboratory Stock
Mri83	F-, DlacU169, araD139, thiA, rpsL, relA, Drbs-7, 'pcnB-'	Lopilato et al. 1986
N1270	Hfr: PO1; pil > pyrB, car-53, leuB6, thr-1, ara-14, folA;TmpR	Lloyd and Thomas 1984
NS388	F-, DEL: lac-proA, his-, trp-,am, thi-, supF81ts, dnaA366am, rpsL;StrR tna::Tn10;TetR, tsx;T6R	Schaus et al. 1981a
P4X	Hfr: PO3; argF > lac, metB1, relA1, l-	Jacob & Wollman 1957
TOE12	thr, leu, proA, his, argE, lac, gal, ara, xyl, mtl, thi, tsxR, rpsl, tonS, thy, rpoB;rifR	K Begg
TP8511	F-, DEL lac-proB, leu-, srlC300::Tn10, TetR, thi-, supE42, fhuA;T1R,Ph80R, recA56	T Paterson, Laboratory Stock

table 2.1: (continued)

STRAIN	GENOTYPE[a]	SOURCE/REFERENCE[b]
TP88	F-, DEL: lac-proB, leu-, thi-, supE42, dnaA46ts, fhuA; T1R, Ph80R, lRB1	T Paterson, Laboratory Stock
TP91	F-, DEL: lac-proB, leu-, thi-, supE42, fhuA; T1R, Ph80R, dnaA+::Tn7; TmpR	T. Paterson. As TP88 but dnaA+ linked to TmpR.
TPK30	leu-, DEL (lac-proB), dnaA46, spoT1::pKN500; KanR, lmb JFL100 lysogen	A. Popplewell, Laboratory Stock
W3110	F-, INV: rrnD-rrnE;1, sup-, l-	Bachmann 1972
W4546	F-, nadC8, galT23	Tritz et al. 1970, CGSC
WM 1152	F-, ilvB/O, lacIqL8, dnaA204ts, l-	W Messer
WM1026	F-, ilvB/O, trp-, lac-, thi-, supD, dnaA46ts	W Messer
WM1029	F-, ilvB/O, trp-, lac-, thi-, supD, dnaA167ts	W Messer
WM1032	F-, ilvB/O, trp-, lac-, thi-, supD, dnaA508ts	W Messer
WT286	F-, DEL: gpt-proA, leu-, thi-1, ara-14, galK2, lacY1, xyl-5, mtl-1, rpsL20;StrR, hsdS20 (rb-, mb-), recA13, l- (ColE1 copy number decreased)	W Tacon; R S Hayward
YA139	Hfr 3000 (PO1 of Hfr H), panB6, relA1, spoT1, thi-, l-	Cronan 1980, CGSC

a. Genotype symbols according to Bachmann (1983)

b. Strains marked GCSC obtained from E.coli Genetic Stock Center.

TABLE 2.2 Bacteriophage

PHAGE	GENOTYPE	SOURCE/REFERENCE
λ115-λ121	*red*$^-$, *gam*$^-$, Δ*att*, Δ*cI*, *Sau*3A or *Eco*RI cut genomic DNA cloned into λEMBL4 (*Eco*RI) or λ2001 (*Sau*3A)	Kohara *et al.* 1987
λ425	*tna*$^+$, *dnaA*$^+$, *srl(1-2)*, *att*, *int*, *imm21*, *ninR5*	Schaus *et al.* 1981a
λ616	*lac5*, *att*$^+$, *imm21*, *cI*$^+$, *ninR5*	Mileham *et al.* 1980
λAO2	λgt11: unidentified *P.falciparum* antigen	G Allan
λNM851	λ46: *polA*$^+$	Laboratory Stock
λRB1	λNF1955: P*dnaA-lacZ*	Braun *et al.* 1985
λ*sidA*	λ616: 8.1kb *groE* insert	Jenkins *et al.* 1986
P1Kc		Newman and Masters 1980
T7/4	Δ genes *1.1* and *1.2*	Richard Hayward

2.3.3 Measurement of optical density

The optical density of cultures was measured using a Perkin-Elmer Coleman Model 55 Spectrophotometer at 540 or 650nm.

2.3.4 Measurement of cell size and cell number

Cell size distribution and cell number were measured using a Coulter Counter Model ZB interfaced with a Coulter Channeliser (Coulter Electronics Ltd., Harpenden, England). 0.2ml samples were mixed with 0.2ml of 0.22μM filtered 20% formaldehyde. Samples (usually 10-100μl) were mixed with 8ml of buffer such that total cell counts were usually in the range 10 000 - 90 000. Dilution buffer consisted of a 0.5% NaCl and 0.08% solution of NaN_3, 0.22μm filtered and made as a 4 litre batch.

TABLE 2.3 Plasmids

PLASMID	DESCRIPTION	SOURCE/REFERENCE
pBC32	AmpR, P*lacUV5-dnaA*, pMB1 replicon	Churchward *et al.* 1983
pBR322	AmpR, TetR, pMB1 replicon	Bolivar *et al.* 1977
pBR325	AmpR, CmpR, TetR, pMB1 replicon	Bolivar 1978
pBR328	AmpR, CmpR, TetR, pMB1 replicon	Soberon *et al.* 1980
pcI^{857}	KanR, *cI*^{857}, p15A replicon	Naumovski and Friedberg 1983
pCM959	*asnA*, *oriC*	Meijer *et al.* 1979
pGW71	AmpR, pOU71 digested with *Bam*HI and *Bgl*II followed by religation. R1 replicon	Larsen *at al.* 1984 (pOU71; pGW71 unpublished)
pHP6	pBR325: AmpS, CmpR, TetR, *oriC* replicon; *Ava*I-*Pst*I Δ replaced with *Ava*I-*Pst*I *oriC* fragment from pCM959	J H Pringle
pHR3	pBR322: AmpR, TetS; 10.1kb HindIII *rpoBC* insert	Newman & Hayward 1980
pJM18	pSR1: AmpR, CmpS, TetS; 7.7kb derivative of pSR1 containing a 3.3kb *Eco*RI-*Bam*HI fragment without the *groE* genes	This work
pJM32	pSR1: AmpR, CmpS, TetS; 8.1kb derivative of pSR1 carrying *groES* and N-terminal *groEL* on 3.7kb *Eco*RI-*Bam*HI fragment	This work
pJM511	pJM513: AmpR, CmpS, TetS, *pcn*$^-$; Δ of 2.6kb *Hin*dIII-*Pvu*II *pcn*$^+$ fragment	This work
pJM513	pBR328: AmpR, CmpS, TetS, *pcn*$^+$; Δ *Eco*RI-*Hin*dIII, replaced with 5.2kb *Eco*RI-*Hin*dIII *pcn*$^+$ fragment from λ115	This work
pJM516	pJM513: AmpR, CmpS, TetS, *pcn*$^+$; 2.5kb internal deletion	This work

TABLE 2.3: (continued)

PLASMID	DESCRIPTION	SOURCE/REFERENCE
pJM87	pJM8701: AmpR, CmpR, *dnaA*$^+$; as pJM8701 but deletion of 1.5kb *Hin*dIII insert	This work
pJM8701	pBR325: AmpR, CmpR, ΔTet; 2.7kb *dnaA* insert, 1.5kb *Hin*dIII insert	This work
pJM88	pUR291: AmpR; Δ*Bam*HI-*Pst*I, insertion of 1.5kb *dnaA* fragment; *lacZ-dnaA* gene fusion	This work
pKN500	KanR, *Eco*RI fragments D and F from R1 *drd-19*, R1 replicon	Molin *et al.* 1979
pND5	pBR325: AmpR, CmpS, TetR; 8.1kb *Eco*RI *groE* insert	Jenkins *et al.* 1986
pPM1000	pBR325: AmpR, CmpR, TetS; insertion of 29.5kb *Hin*dIII *E.coli* terminus fragment	I R Oliver
pPM30	AmpR, *par*$^+$, pSC101 replicon	Meacock & Cohen 1980
pPM4000	pBR325: AmpR, CmpS, TetR; insertion of 6.3kb *Eco*RI *E.coli* terminus fragment	I R Oliver
pSR1	pND5: AmpR, TetR; Tn1725 (CmpR) inserted into 8.1kb *groE* fragment	J H Pringle
pUR291	AmpR, P*lacUV5-lacZ*, pMB1 replicon	Ruther & Muller-Hill 1983
pVH1	KanR, *lacI*^{q}, ColD replicon	M Bagdasarian

2.3.5 Testing of UV sensitivity of *recA* and *polA* strains

A single colony of the strain to be tested was streaked across an L-Agar plate using a sterile toothpick. In addition, single colonies of a known *recA* or *polA* strain and a wild-type control were streaked across the plate. The plate was covered with a piece of card and exposed to successively longer periods of UV; commonly 0, 10, 30 and 60 seconds, under an ultraviolet lamp calibrated at 600 ergs mm^2 sec^{-1}. Growth between streaks was then compared for the different time intervals, and an estimate of UV sensitivity made.

2.3.6 Hfr mapping

An overnight culture of the Hfr donor strain was diluted 1:100 into fresh L-Broth and grown at 37°C with gentle shaking until an $O.D._{540}$ of 0.5 was achieved. Recipient cells were grown in L-Broth at 37°C with vigorous shaking until late log phase. 10mls of the Str^s donor culture was mixed with 1ml of the Str^r recipient strain and left to stand at 37°C for 60 minutes. As a control, 10mls of donor was mixed with 1ml of broth; similarly 1ml of recipient was mixed with 10mls of broth. The mixture was then vortexed vigorously for 1 minute and dilutions of 10^0, 10^{-1} and 10^{-2} were plated on minimal media selective for various transferred markers and containing streptomycin to select against donor growth. Exconjugants were purified by patching onto plates of the same medium prior to growing up in liquid media and testing for coinheritance with the marker to be mapped.

2.3.7 Hfr plate mating

A single colony of the recipient strain was used to innoculate 5mls of L-Broth and grown until late log phase. A loopful of this was then streaked across a minimal media plate containing streptomycin and selective for a particular nutritional marker. Across this and at right angles to it was streaked a single freshly growing colony of the Hfr donor strain. The plates were incubated at 37°C and examined for evidence of colony growth at the juncture of the two streaks. If required, colonies were purified on plates of the same type prior to testing for coinheritance of a particular marker.

2.3.8 Preparation of *E.coli* competent cells

A fresh overnight culture of the strain (grown from an isolated colony and under selection, if appropriate) was used to innoculate L-Broth containing 20mM $MgCl_2/SO_4$ (equimolar mixture) by diluting 1:50 (v:v), and incubated at 37°C (30°C for *ts* strains) with vigorous shaking until the OD_{540} was between 0.5-0.6. The culture was chilled on ice, transferred to sterile centrifuge bottles and pelleted at 5000rpm for 10mins at 4°C. The supernatant was discarded, and the cells were resuspended in $^1/_4$ the original culture volume of ice-cold 0.1M $MgCl_2$. The cells were again pelleted, and resuspended in $^1/_{20}$ the original volume of ice-cold 0.1M $CaCl_2$. The cell suspension was then maintained on ice for about 45mins. The cells were pelleted and resuspended in the same volume of 0.1M MOPS (3-N[N-Morpholino]propanesulfonic acid; Sigma) pH 6.5, 50mM $CaCl_2$, 20% glycerol. Cells were then either used immediately for transformation or stored at -20°C in small aliquots until required.

TABLE 2.4 Growth media, phage and bacterial buffers

Growth Media

L-Broth (LB):	Difco Bacto Tryptone	10g
	Difco Bacto Yeast Extract	5g
	NaCl	5g
	pH to 7.2 with NaOH; distilled water to 1 litre	
L-Broth Agar:	L-Broth + 15g Difco agar per litre	
LB Top Agar:	L-Broth + 6.5g Difco agar per litre	
VB Minimal Media:	20x VB Salts	25ml
	20% carbon source	5ml
	Supplements as required	
	Distilled water to 1 litre	
VB Minimal Agar:	As VB Minimal Media + 15g Difco agar per litre	
20x VB Salts:	$MgSO_4.7H_2O$	4g
	Citric acid	40g
	KH_2PO_4	400g
	$NaNH_4.HPO_4.4H_2O$	70g
	Distilled water to 1 litre	
M9 Glucose:	4x M9 Salts	100ml
	20% glucose	4ml
	1M $MgSO_4$	0.4ml
	1mg ml^{-1} Thiamine HCl (B_1)	0.8ml
	Distilled water to 1 litre	
M9 Maltose:	As M9 Glucose but contains 0.2% maltose instead	
4x M9 Salts:	Na_2HPO_4	24g
	KH_2PO_4	12g
	NH_4Cl	4g
	NaCl	2g
	Distilled water to 1 litre	

Buffers

Phage Buffer:	Na_2HPO_4	7g
	KH_2PO_4	3g
	NaCl	5g
	$MgSO_4$ (0.1M)	10ml
	$CaCl_2$ (0.1M)	10ml
	1% gelatin solution	1ml
	Distilled water to 1 litre	
Bacterial Buffer:	KH_2PO_4	3g
	Na_2HPO_4	7g
	NaCl	4g
	$MgSO_4.7H_2O$	2g
	Distilled water to 1 litre	

TABLE 2.5 Amino acid, Purine, Pyrimidine, and Vitamin Concentrations of Stock Solutions for Supplementation of Minimal Media

	(1)	(2)	(3)	(4)	(5)	(6)
AMINO ACIDS						
DL-Alanine	ALA	H_2O	10	A	5	100
L-Arginine HCl	ARG	"	2	A	5	20
L-Asparagine	ASN	"	10	A	5	100
L-Aspartic acid	ASP	"	10	A	5	100
L-Cysteine HCl [a]	CYS	"	2	A	5	20
L-Glutamic acid	GLT	"	10	A	5	100
L-Glutamine	GLN	"	10	A	5	100
Glycine	GLY	"	10	A	5	100
L-Histidine HCl [b]	HIS	"	2	A	5	20
L-Isoleucine	ILE	"	2	A	5	20
L-Leucine	LEU	"	2	A	5	20
L-Lysine HCl	LYS	"	10	A	5	100
DL-Methionine	MET	"	2	A	5	20
L-Phenylalanine	PHE	0.001N NaOH	2	A	5	20
L-Proline	PRO	H_2O	3	A	5	30
DL-Serine	SER	"	10	A	5	100
DL-Threonine	THR	"	10	A	5	100
L-Tryptophan [b]	TRP	"	2	A	5	20
L-Tyrosine	TYR	0.01N NaOH	2	A	5	20
DL-Valine	VAL	H_2O	4	A	5	40
L-Isoleucine/DL-Valine	ILV	"	2/4	A	5	20/40
Casamino acids	CAA	"	100	A	25	5000
PURINES AND PYRIMIDINES						
Adenine	ADE	0.03N HCl	2	A	5	20
Thymine	THY	H_2O	2	A	2.5	10
					10	40
Uracil	URA	"	2	A	5	20
VITAMINS						
Biotin	BIO	H_2O	0.1	F	2.5	0.5
Niacin (nicotinic acid)	NAD	"	0.5	F	1	1
Thiamine HCl (B_1) [b]	THI	"	1	F	1	2

(1) Abbreviation
(2) Solvent
(3) Concentration of stock solution (mg ml^{-1})
(4) Method of sterilization (A: autoclave; F: filter)
(5) Amount of stock solution added to medium (ml 500ml^{-1} medium)
(6) Final concentration in medium (ug ml^{-1})

NOTES:

a. Cysteine should be freshly prepared (at least every week), since it is rapidly oxidized in solution to the highly insoluble cystine.

b. Solutions should be stored in dark or covered bottles.

TABLE 2.6 Stock Solutions of Antibiotics and Other Selection Agents

	(1)	(2)	(3)	(4)	(5)	(6)	(7)
Ampicillin [a]	Amp	H_2O	100	F	−20°C	10 50	0.05 0.25
Chloramphenicol [b]	Chl	ethanol	20	-	−20°C	30	0.75
Kanamycin sulphate	Kan	H_2O	25	F	−20°C	20 50	0.40 1.00
Nalidixic acid	Nal	0.1N NaOH	20	F	−20°C	20	0.50
Rifampicin [c]	Rif	dimethyl-formamide	100	-	−20°C	100	0.50
Spectinomycin dihydrochloride	Spc	H_2O	50	F	−20°C	25	0.5
Streptomycin sulphate	Str	H_2O	100	F	−20°C	200	1.00
Tetracycline hydrochloride [d]	Tet	50% ethanol	10	-	−20°C dark	5 10 15	0.25 0.50 0.75
Trimethoprim	Tmp	methanol	5	-	4°C	10 50	1.00 5.00
5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside	X-gal	dimethyl-formamide	20	-	−20°C dark	20 40	0.50 1.00
Isopropyl-β-D-thiogalactopyranoside	IPTG	H_2O	20	-	−20°C	2	0.05

(1) Abbreviation
(2) Solvent
(3) Concentration of stock solution (mg ml^{-1})
(4) Method of sterilization
(5) Storage conditions
(6) Final concentration in medium (mg ml^{-1})
(7) Amount of stock solution added to medium (ml $500ml^{-1}$ medium)

NOTES:

a. Discard plates after storage at 4°C for 2 weeks.

b. Free chloramphenicol should be used NOT the succinate salt, which is less effective. Discard plates after storage at 4°C for 1 week.

c. Make plates fresh and use within 2 days.

d. Mg^{2+} ions are antagonistic to activity; use in media low in magnesium. Store plates at 4°C in the dark.

2.3.9 Purification of minicells for *in vivo* protein synthesis studies

Minicells were purified and labelled with ^{35}S-methionine by the method of Reeve (1977). 8 sucrose gradients were prepared by freezing 35ml aliquots of 20% (w:v) sucrose in M9-glucose medium (table 2.4) in 50ml polycarbonate centrifuge tubes (-70°C), and allowing the frozen solutions to thaw undisturbed at 4°C overnight. Transformants of the minicell producing strain DS410 were used to innoculate a 2 litre overnight culture in L-broth containing antibiotic, the cells being grown until early stationary phase was reached. The majority of the parental cells were removed from the culture by centrifugation at 2500rpm (900 x g_n) in a Sorvall GS-3 rotor for 15 mins at 2°C in 500ml bottles. The supernatant was transferred into fresh 500ml bottles, and the minicells and remaining parental cells were harvested by centrifuging the supernatant at 8 500rpm (10 000 x g_n) for 20 mins. The supernatant was poured off except for about 2.5mls which was left in each bottle and used to resuspend the minicell pellets by sucking the liquid in and out of a sterile Pasteur pipette several times. The suspension was transfered to a 30ml Corex tube on ice and whirlimixed vigorously so that the aggregated minicells were fully dispersed. About 2.5ml of the minicell suspension was layered onto each of 4 x 35ml sucrose gradients and centrifuged at 5 000rpm (2 700 x g_n) in a Sorvall HB-4 rotor (swing-out) for 20 mins at 2°C. The minicell bands were collected from the gradients using a 20ml syringe and 19G x 4cm hypodermic needle which had been bent to a right angle at the tip. The minicells banded in the upper half of the gradients, and the top two-thirds only were removed (approximately 6-10ml per gradient) in order to minimize parental cell contamination. Samples collected from pairs of gradients were pooled, and the minicells pelleted by centrifugation in a Sorvall SS-34 rotor at 13 000rpm (15 600 x g_n) for 10 mins at 2°C. The supernatant was discarded and both minicell pellets were resuspended in a pooled total of 4ml of M9-glucose medium by whirlimixing vigorously as before. About 2ml of the minicell suspension was layered onto each of 2 x 35ml sucrose gradients. These were centrifuged, harvested, pooled and finally pelleted as previously. This pellet was resuspended in 2ml of M9-glucose and layered onto 1 x 35ml sucrose gradient. This was centrifuged and the band collected as before, followed by pelleting of the minicells in a Sorvall SS-34 rotor at 8 000rpm (6 000 x g_n) for 10 mins at 2°C.

The minicells were resuspended in 1ml of 30% (w:v) glycerol in M9-glucose medium and aliquoted into 1.5ml Eppendorf tubes such that the volume of each aliquot when diluted to 1ml gave an OD_{600} of 0.2, which is equal to approximately 2x 10^9 minicells:

$$\text{Volume of aliquot (usually 30-100ul)} = \frac{0.2 \times 100}{O.D._{600}}$$

The cells were stored at -70°C until required.

2.4 Phage techniques

2.4.1 Preparation of λ plate lysates

Cells were grown in L-Broth + 20mM $MgSO_4$ and maltose at 0.2% until mid log phase. 0.2ml aliquots were then mixed with 10^6 phage, incubated at 37°C for 5 minutes, and 3ml of LB Top Agar containing 20mM $MgSO_4$ and 0.2% maltose was added. This was poured onto a pre-warmed L-Broth plate, left to set, and the plate incubated at 37°C for 8-9 hours until visible lysis had occured. 5 ml of L-Broth was pipetted onto the plate and removed along with the top agar into a half ounce bottle. 50µl of chloroform was added and the bottle vortexed vigorously for 30 seconds. The debris was removed by spinning in a bench centrifuge. The supernatant was transferred to a fresh bottle and a few drops of chloroform were added to prevent microbial growth.

2.4.2 UV induction of λ lysogens

Lysogenic bacteria were grown with maximum aeration in L-Broth + 20mM $MgSO_4$ at 37°C until an $O.D._{540}$ of 0.3 was achieved. The cells were harvested by centrifugation and resuspended in half the original volume of 20mM $MgSO_4$. This was transferred to a sterile glass petri dish and the cells exposed to 600ergs mm^2 of UV light before being diluted into 5 times the volume of fresh L-Broth + 20mM $MgSO_4$. The culture was then grown at 37°C with maximum aeration until lysis occurred. A few drops of chloroform were added, and the lysate was clarified by centrifugation prior to titration.

2.4.3 Thermal induction of λ lysogens

Lysogenic bacteria were grown with maximum aeration in L-Broth + 20mM $MgSO_4$ at 30°C until an $O.D._{540}$ of 0.3 was achieved. The culture was transferred to a 42°C water bath and shaken for 20 minutes to induce the prophage. It was then transferred to 37°C and shaken vigorously until lysis occurred, after which chloroform was added and the lysate clarified as above.

2.4.4 Amplification of λ by liquid infection

Cells were grown with maximum aeration at 37°C in L-Broth + 20mM $MgSO_4$ + 0.2% maltose until an $O.D._{540}$ of 0.2 was achieved. Cell density at this O.D. was assumed to be 2 x 10^8 per ml, and phage were added such that a multiplicity of infection (m.o.i.) of 0.5 was achieved. The culture was shaken vigorously at 37°C for a further 2-3 hours or until visible lysis had occurred, after which it was treated as above.

2.4.5 Selection and testing of λ lysogens

Recipient cells were grown at 30°C or 37°C in L-Broth + 20mM $MgSO_4$ + 0.2% maltose until an $O.D._{540}$ of between 0.1-0.5 was achieved. Depending on the strain and the marker to be tested different amounts of cells had to be plated in order to achieve satisfactory complementation. For nutritional markers 0.1-0.3ml of cells were spread directly onto a minimal plate and allowed to dry. For antibiotic resistance/ temperature resistance etc., 0.1-0.3ml of cells were mixed with 3ml of LB Top Agar + 20mM $MgSO_4$ and poured onto an L-Broth (+ antibiotic if required) plate and left to set. Phage to be tested (+ a control phage) were spotted onto the plate. Colonies which grew under selection were streaked to purity (maintaining selection), grown up, and the supernatant tested for the presence of phage. This latter step was only necessary for markers which exhibited a poor selectable phenotype.

2.4.6 Preparation of P1 plate lysates

Basically as per preparation of phage λ plate lysates except 2.5mM $CaCl_2$ was added instead of 20mM $MgSO_4$ and the maltose was omitted. Particular nutitional requirements of strains unable to grow on L-Broth alone were also met.

2.4.7 Amplification of P1 by liquid infection

Cells were grown with maximum aeration at 37°C (or 30°C if the strain was temperature sensitive) in L-Broth + 2.5mM $MgSO_4$ + particular nutritional additives as required by the strain. When an $O.D._{540}$ of 0.2 had been achieved cells were infected with P1 at an m.o.i. of 0.5 as above. Shaking at 37°C was continued for 4-5 hours or until visible lysis had occurred, after which chloroform was added and the lysate cleared by centrifugation. The lysate was then titrated.

2.4.8 P1 transduction

A fresh overnight culture of the recipient cells grown to stationary phase in L-Broth + nutritional requirements/antibiotics as required was used. This was pelleted in a bench centrifuge and resuspended in $^1/_{10}$ the volume of 10mM Tris-HCl 10mM $CaCl_2$ pH 8.0. 0.1ml aliquots were transferred to 1.5ml eppendorf tubes and 10^8 phage were added, usually in 0.1ml also. This was incubated at 37°C (or 30°C for temperature sensitive strains) for 15 minutes, the cells pelleted, and the supernatant discarded. The cells were then resuspended in 1ml of either phage buffer (nutritional marker) or L-Broth + 10mM trisodium citrate (antibiotic resistance marker). When selecting for nutritional markers the cells were plated straight away; for antibiotic resistance markers, expression time of 45 minutes (37°C) or 60 minutes (30°C) was allowed.

2.5 β-galactosidase enzyme assays

The method was essentially that of Miller (1972). For assays of β-galactosidase under control of the *dnaA* promoter where a chromosomal copy of the *lacZ* gene existed, glucose at 0.2% was added to the medium to minimise transcription from this source. Otherwise the cells were grown in L-Broth at the specified temperature. If induction of β-galactosidase under control of its own promoter was required, IPTG (isopropyl-β-D-thiogalactoside) was added at 4-20μg ml^{-1}, depending on whether a single or multiple copies of the gene were present. The growth of the culture was followed by measuring the $O.D._{540}$. Regular dilutions (using pre-warmed media) were made such that the $O.D._{540}$ of the culture was kept between 0.1-0.2 prior to sampling.

All assays were carried out in 1.5ml eppendorf tubes. Paired samples of 0.5ml of culture were taken and added to 0.5ml of Z buffer, increasing the buffer ratio for higher enzyme activities. To permeablize the cells the Z buffer contained 0.005% SDS; in addition, 15μl of chloroform was added to each sample. The sample was vortexed for 15 seconds and 200μl of 4mg ml^{-1} ONPG (o-nitrophenyl-β- D-galactoside) in 0.1M MOPS pH 7.0 was added. The sample was incubated at 28°C until sufficient yellow colour (o-nitrophenol) had appeared such that when 0.5ml of Na_2CO_3 was added to stop the reaction, the absorbance at 420nm was between 0.4-2.0. (Standard curves constructed in this laboratory by T. Paterson had demonstrated that the absorbance measurements were linear within this range). Instead of measuring samples at both 420nm (o-nitrophenol) and 550nm (cell scattering), it was found more convenient to remove cell debris by spinning in a bench top centrifuge for 5 minutes.

Total enzyme activity was calculated as:

$$1000 \times \frac{O.D._{420}}{\text{time of incubation (min.)} \times \text{dil. of sample}}$$

Specific enzyme activity was expressed as **Miller Units**:

$$\frac{\text{enzyme activity}}{O.D._{600} \text{ of the culture at time of sampling}}$$

i.e. Enzyme units per cell mass.

Z buffer was made as follows, and stored at 4°C until use.

Per litre:-

16.1g	$Na_2HPO_4.7H_2O$	0.06M
5.5g	$NaH_2PO_4.H_2O$	0.04M
0.75g	KCL	0.01M
0.246g	$MgSO_4.7H_2O$	0.001M
2.7ml	β-mercaptoethanol	0.05M

pH adjusted to 7.0, 0.22µM filter sterilised.

2.6 Measurement of DNA by fluorometric assay

The method used was essentially that described by Legros and Kepes (1985). Cells were grown in L-Broth + antibiotic if required at the specified temperature. The growth of the culture was followed by measuring the $O.D._{540}$, and regular dilutions (using pre-warmed media) were made in order to maintain steady state growth. Cultures were maintained at an $O.D._{540}$ of 0.1-0.2 until the growth rate had stabilised for at least 90 minutes before samples were taken and temperature shifts could be performed as required.

1ml samples were transferred into 1.5ml eppendorf tubes, simultaneously measuring the $O.D._{540}$ of the culture. These were pelleted in a bench centrifuge and washed three times in 1ml of bacterial buffer (Table 2.4), being resuspended to a final volume of 1ml. The $O.D._{540}$ was measured again. The culture was then mixed with 2mls of a DAPI (4'6-Diamidino-2- phenylindole.2HCl; Sigma) solution at 140ng ml^{-1}, and 30µl of toluene was added to permeablise the cells. Fluorescence was measured using a KONTRON SFM25 fluorimeter equipped with a 150w Xenon high pressure lamp and an R212 photomultiplier, using excitation and emmission wavelengths of 346nm and 452nm respectively. Voltage calibration was set at 380v. Entrance and exit slits for the two monochromators were 10 and 15 nm respectively. DNA content was

expressed either as total DNA (fluoresence) per sample, or specific content, in which case the total fluorescence was divided by the **washed** $O.D._{540}$ of the sample.

2.7 Fluorography of whole fixed cells

(Protocol supplied by S. Hiraga). Samples of bacteria (200µl) were fixed in the same volume of 20% (v:v) formaldehyde in a 1.5ml eppendorf tube. The cells were pelleted by centrifugation and washed 3 times in 0.85% (w:v) NaCl, being resuspended in a final volume of 20µl. This was spotted onto a glass microscope slide which had been prepared by washing in Nitric Acid followed by rinsing in distilled H_2O. The spot was allowed to dry at room temperature. This was overlaid with 30µl of methanol and allowed to dry for 5 minutes at room temperature. The glass slide was then washed by alternately submerging and removing it from a beaker of **tap** water about 6 times. The slides were placed on tissue paper and allowed to dry. 10µl of a 5µg ml^{-1} solution of Poly-L-lysin (Sigma) was placed over the bacterial spot and gently spread using the end of a plastic tip. This was allowed to dry whereopon 10µl of a 5µg ml^{-1} solution of DAPI was added to the spot. The bacterial sample was then covered with a clean glass cover slip and examined under phase contrast and ultraviolet light using a Leitz Vario-Orthomat Microscope.

2.8 DNA techniques

2.8.1 Small scale purification of λ DNA

A fresh overnight culture which had been grown in L-Broth + 20mM $MgSO_4$ + 0.2% maltose was used. To 0.1ml of this culture was added 10^8 phage followed by 4ml of L-Broth supplemented as above. The culture was shaken at 37°C for 3-5 hours until lysis had occurred (this was most easily seen by comparison with a culture lacking phage). 50µl of chloroform was added, the mixture vortexed, and the debris removed by spinning in a bench centrifuge. The supernatant was transferred to a sterile half ounce bottle, and 10µl of a 10mg ml^{-1} solution of RNAase-A and DNAase-1 (Sigma) was added. This was then shaken at 37°C for 30 minutes. 4mls of a solution containing 20% w/v PEG 6000, 2M NaCl in phage buffer was added to a 15ml Corex Tube followed by the treated lysate. This was left to stand for one hour (or overnight if more convenient) at 4°C, followed by centrifugation for 20 minutes at 10 000rpm, 4°C in a Sorvall Superspeed Centrifuge. The supernatant was removed by aspiration, leaving as little of the PEG solution as possible. The phage pellet was gently resuspended in 0.5ml phage buffer, transferred to a 1.5ml eppendorf tube, and 0.5ml of chloroform was added. The tube was vortexed in 3 brief bursts and spun in a micro

centrifuge for 1 minute. The upper aqueous layer was carefully transferred to a new eppendorf tube, being careful not to disturb the thin white pellet at the interface (mostly PEG and protein). 500µl of phenol equilibrated with T.E. (10mM Tris-HCl pH 8.0, 1mM EDTA) was added, followed by 100µl of T.E. The two layers were mixed by inversion, and the tube spun for 2 minutes in a micro centrifuge. 500µl of the upper aqueous layer was transferred to a fresh tube and to this was added 500µl of phenol:chloroform (1:1) equilibrated with T.E. This was spun for 2 minutes in a micro centrifuge, 450µl of the aqueous layer was transferred to a fresh eppendorf, and 450µl of chloroform:iso-amyl alcohol (24:1) was added. It was then mixed and spun as above, 400µl being transferred to a fresh eppendorf, and 800µl of ethanol (room temperature) being added. The tube was left on ice for 5 minutes, followed by centrifugation in a micro centrifuge for 15 minutes at 4°C. The supernatant was removed and the pellet washed in 70% ethanol (-20°C), then dissolved in 400µl of T.E. To this was added 40µl of 3M NaAcetate, followed by 800µl of ethanol. THis was mixed well, left on ice for 5 minutes, and the tube spun at 4°C as before. The supernatant was removed, and the pellet washed in 70% ethanol. It was then dried under vacuum, prior to being dissolved in 50µl of T.E. The DNA was stored at -20°C until required. This protocol gave a yield of between 5-10µg of DNA per lysate. (Protocol supplied by Annette Campbell).

2.8.2 Purification of plasmid DNA Birnboim-Doly minipreps

The method used is based upon that described by Birnboim and Doly (1979). The protocol was modified to reduce the time required for several steps without noticably affecting either yield or purity of the plasmid product. The solutions used in this protocol are shown listed below:-

Resuspension buffer:	Tris-HCl pH 8.0	25mM
	EDTA	10mM
	Glucose	50mM
Alkaline SDS solution[1]:	NaOH	0.2M
	SDS	1% w:v
High salt solution:	Na Acetate pH 5.0	3.0M
Low salt solution:	Na Acetate pH 6.0	0.1M

[1] The alkaline SDS solution was made freshly immediately before use.

1.5ml of a plasmid containing stationary phase overnight culture was transferred to an eppendorf tube and the cells pelleted in a microcentrifuge. The

supernatant was discarded and the cells resuspended in 0.1ml of resuspension buffer by vortexing. 0.2ml of alkaline SDS solution was added and the suspension was left for 5 minutes on ice, mixing occasionally by inversion of the tube. 0.15ml of high salt solution was then added and the tube left on ice for 5 minutes with mixing as above. It was then centrifuged at 4°C in a microcentrifuge for 10 minutes and the supernatant transferred to a fresh eppendorf tube. 1ml of ethanol was added and the tube was placed on ice for 5 minutes. The precipitated nucleic acids were pelleted by centrifugation in a microcentrifuge for 2 minutes. The supernatant was discarded and the pellet resuspended in 0.2ml of low salt solution. 0.4ml of ethanol was added and the plasmid preparation was placed on ice and pelleted as before. The pellet was washed in 70% ethanol (-20°C), prior to vacuum dessication and resuspension in 50µl of T.E. (10mM Tris-HCl pH8.0, 1mM EDTA). DNA prepared in this way was found to remain stable for several months at 4°C.

2.8.3 Large scale preparation of plasmid DNA

For large scale preparations of highly purified plasmid DNA, caesium chloride/ ethidium bromide equilibrium density centrifugation was found to give the most satisfactory results. The solutions used are shown below:-

T.E.: 10mM Tris-HCl, pH 8.0
1mM EDTA

Resuspending buffer: 50mM Tris-HCl, pH 8.0
25%(w:v) sucrose

Lysozyme solution: 20mg ml^{-1} in H_2O

EDTA: 0.5M EDTA (ethylenediaminetetraacetic acid), pH 8.0

RNase A solution: 10mg ml^{-1} RNase A (heat treated to inactivate DNase)

Triton lysis buffer: 50mM Tris-HCl, pH 8.0
62.5 mM EDTA
0.1%(w:v) Triton X-100

Ethidium bromide solution: 10mg ml^{-1} in T.E.

The plasmid carrying strain was grown from a single colony in 500ml of L-Broth to stationary phase maintaining selection throughout. The culture was chilled on ice and pelletted in a 500ml polypropylene bottle at 4°C. The supernatant was discarded and the pellet resuspended in 200ml of ice-cold T.E. The cells were pelletted as before and the supernatant removed. The pellet was resuspended in 5ml

of resuspending buffer, then transferred to a 50ml polypropylene centrifuge tube. 1ml of a 20mg ml^{-1} solution of lysozyme was added, the suspension was left on ice for 5 minutes, then 1ml of 0.5M EDTA (pH 8.0) followed by 0.8ml of the RNase A solution. This was mixed well and left on ice for 5 minutes. 9ml of Triton lysis buffer was then added, the tube covered with parafilm, and the lysate mixed by inversion while being kept on ice for 10 minutes. It was then centrifuged at 15 000 rpm for 30 minutes at 4°C to pellet the cellular debris and most of the chromosomal DNA. The cleared lysate was then transferred to a sterile glass measuring cylinder, and T.E., ethidium bromide solution and caesium chloride were added to give a final density of 1.55g ml^{-1} and ethidium bromide concentration of 200µg ml^{-1} (17.1g CsCl + 0.342ml of EtBr solution made up to 23ml with T.E.). This was pipetted into two 11.5ml Sorvall Polyallomer Crimp Seal Tubes, balanced to within 0.05g, and centrifuged in a Beckman 50Ti fixed angle rotor at 40 000rpm for 60 hours at 20°C. The DNA was visualised under UV light, and the upper (plasmid) band was removed using a 2ml syringe and 19 gauge needle by puncturing the side wall of the tube just below the DNA band. After extraction this was made up to 11.5ml using a stock solution of CsCl and EtBr of density and concentration as above and the centrifugation run repeated. The plasmid band was removed as before and transferred to one or more eppendorf tubes. Ethidium bromide was removed by extracting 3 times with an equal volume of iso-amyl alcohol (equilibrated with a saturated solution of CsCl), and the solution dialysed against 4 changes of 500ml T.E., 4°C, for 24 hours to remove the CsCl.

2.8.4 Purification of plasmid DNA by use of NACS™ chromatography

Where small amounts of rapidly purified plasmid DNA were required, it was found more convenient to use NACS.52 columns (BRL Inc.) to clean up Birnboim-Doly miniprep DNA (see above) rather than CsCl/EtBr equilibrium centrifugation. Since the salt concentration is critical to the success of this procedure, all solutions were prepared in a volumetric flask to minimise inaccuracies.

The columns were hydrated with 2.0M NaCl in T.E. (pH 7.2) as per manufacturers instructions. The resin was then equilibrated by washing 3 times in 0.2M NaCl in T.E. (pH 7.2), followed by loading of the nucleic acid sample under gravity. The sample was prepared as per Birnboim-Doly minipreps but with the following modifications. Instead of resuspending the dessicated pellet in 50µl of T.E., it was dissolved in 500µl of 0.2M NaCl in T.E. (pH 7.2), and 20 units of RNase T1 was added. This was left for 15 minutes at 37°C before loading onto the column. The resin was subsequently washed with 5-10ml of 0.5M NaCl in T.E. (pH 7.2) to remove RNA,

proteins etc., prior to elution, under gravity, of the plasmid in 2 times 0.25ml of 0.7M NaCl in T.E. (pH 7.2). These were pooled, 1ml of ethanol was added, the sample left on ice for 5 minutes, followed by centrifugation at 4°C for 20 minutes in a microcentrifuge. The supernatant was discarded and the pellet washed in 70% ethanol (-20°C), prior to vacuum dessication and resuspension in 25µl of T.E.

2.8.5 Ethanol precipitation of DNA

$^1/_{10}$ volume of 3M Na Acetate was added to the DNA solution in T.E., followed by 2 volumes of ethanol in a 1.5ml eppendorf tube. (e.g. 0.2ml DNA solution + 20µl 3M Na Acetate + 440µl of ethanol). This was left on ice for 5 minutes, before centrifugation in a microcentrifuge for 30 minutes at 4°C. The supernatant was discarded and the pellet washed in 1ml of 70% ethanol (-20°C) prior to vacuum dessication.

2.8.6 Elution of specific DNA fragments from agarose gels using BioTrap apparatus

The apparatus was assembled as per manufacturers instructions (Schleicher & Schuell GmbH.), such that the minimal 'trapchamber', corresponding to a volume of about 200µl was obtained. The gel slice containing the DNA fragment of interest was placed in the elution chamber containing about 10ml of Tris Acetate gel buffer (Table 2.8), and the Biotrap placed in a gel tank containing the same buffer. A perspex block was placed between the Biotrap and the free wall of the gel tank to reduce the free buffer area, and so minimise heating of the buffer and excessive current flow. A voltage of 150-200 volts was applied for 8-10 hours (or overnight if more convenient) while the DNA migrated into the 'trap' chamber. The current was reversed for 20 seconds to move the DNA away from the outer membrane, and then switched off. The DNA in buffer was removed using a pasteur pipette and concentrated by ethanol precipitation. Yields of between 80-90% were commonly obtained.

2.8.7 Elution of specific DNA fragments from agarose gels using GENECLEAN kit

Use of Geneclean (BIO 101 Inc., CA) was found to be most effective for the elution of small quantities of DNA from gel slices. The procedure was as recommended by the manufacturers. The gel slice was weighed, placed in a 1.5ml eppendorf tube and 2-3 volumes of the NaI stock solution was added. The tube was placed at 50°C for 5 minutes and 5µl of the Glassmilk suspension added. This was mixed and incubated at room temperature for 5 minutes. The Glassmilk/DNA complex

TABLE 2.7 Buffers for Restriction Endonuclease Digestion and Ligation of DNA

	Working Concentration of Constituents	10x Buffer
Restriction Buffers		
Universal Type 1:		
20mM	Tris-HCl, pH 7.5	0.20M
50mM	NaCl	0.50M
6mM	$MgCl_2$	0.06M
0.01%	BSA (bovine serum albumen)	0.1%
(10mM	2-mercaptoethanol	0.10M) [a]
Universal Type 2:		
33mM	Tris-acetate, pH 7.9	0.33M
66mM	CH_3COOK	0.66M
10mM	CH_3COOMg	0.10M
0.01%	BSA	0.1%
(10mM	2-mercaptoethanol	0.10M) [a]
***Eco*RI Type:**		
100mM	Tris-HCl, pH 7.5	1.00M
50mM	NaCl	0.50M
6mM	$MgCl_2$	0.06M
0.01%	BSA	0.1%
***Bam*HI/*Sal*I Type:**		
10mM	Tris-HCl, pH 8.0	0.01M
150mM	NaCl	1.50M
6mM	$MgCl_2$	0.06M
0.01%	BSA	0.1%
(10mM	2-mercaptoethanol	0.10M) [a]
Ligation Buffer [b]		
75mM	Tris-HCl, pH 7.8	0.75M
20mM	$MgCl_2$	0.20M
1mM	spermidine tri-HCl	0.01M
0.01%	BSA	0.10%
20mM	DTT	0.20M
1mM	ATP	0.01M

a. 2-mercaptoethanol is added to the 10x buffer solution just before use; add 3ul of 10x diluted stock solution to each 40ul aliquot of 10x restriction buffer. 10x buffer solution with 2-mercaptoethanol added can be stored frozen at -20°C for 3-4 weeks without deterioration.

b. The complete 10x ligation buffer is stored in 20ul aliquots at -20°C and need only be thawed and mixed before use. Unused buffer can be refrozen and stored at -20°C without deterioration.

was pelleted by spinning in a microcentrifuge for 5 seconds, the supernatant discarded and the pellet washed 3 times in NEW. After the third wash as much NEW as possible was removed and the pellet was eluted into 20µl of T.E. Yields of 90–95% were obtained.

2.8.8 Restriction of DNA

DNA was digested with restriction enzymes at 37°C, usually for 1 hour, and in a volume of 10–20µl. The buffers used are shown in table 2.7. Enzymes were obtained either from BCL Ltd. or Boehringer Mannheim GmbH. *Bam*HI was digested in *Bam*HI/*Sal*I type; *Hin*dIII in Universal type 2; *Eco*RI in *Eco*RI type; and *Acc*I, *Ava*I, *Hae*II, *Hin*cII, *Hin*fI, *Pst*I and *Pvu*II in Universal type 1. A two to fivefold excess of the restriction enzyme was commonly added. Where appropriate the enzyme was inactivated by heat killing at 70°C for 10 minutes, or in the case of *Bam*HI, by phenol extraction followed by ethanol precipitation. In the case of λ digests this step was also necessary to melt the cohesive ends prior to gel electrophoresis.

2.8.9 Filling in of protruding 5′ ends

Protruding 5′ ends produced by restriction digests were filled in using the Klenow fragment of *E.coli* DNA polymerase I (Boehringer Mannheim GmbH.). The reaction mixture was as follows:–

Restriction fragment (1–2µg in H_2O)	5–20µl
2mM solution of all 4 dNTPs	1µl
10x nick-translation buffer[1]	2.5µl
H_2O	**to 25µl**

[1] Nick-translation buffer (x10) 0.5M Tris–HCl pH 7.2
0.1M $MgSO_4$
1mM dithiothreitol
500µg ml^{-1} BSA

To this was added 2 units of Klenow followed by incubation at room temperature for 30 minutes. The Klenow was inactivated by heating to 75°C for 10 minutes followed by transfer to an ice bath. The DNA was added to a ligation reaction without further purification.

2.8.10 Phosphorylation of 5'-hydroxyl ends

The linkers used in this work were supplied with 5'-hydroxyl ends. T4 DNA Kinase (Boehringer Mannheim GmbH.) was used to convert the ends to 5'-phosphates and thus allow the linkers to be ligated to the fragment of interest. The reaction was set up as follows:-

Linkers in H_2O	25µl (5µg)
10x ligation buffer[1]	3µl
2 units of T4 DNA Kinase	2µl
	30µl total

[1] Shown in table 2.7

The mixture was incubated at 37°C for one hour and then stored at -20°C until required. Further treatment was unnecessary, and the DNA was added directly to the ligation mix.

2.8.11 Ligation of DNA fragments

Ligation reactions were performed at a **final** DNA concentration of between 10-50ng μl^{-1}. $^1/_{10}$ volume of 10x ligation buffer (table 2.7) was added to the reaction mix followed by 2-10 units of T4 DNA ligase (New England Biolabs Inc.), depending on the amount of DNA present. Blunt ended ligations were performed overnight at 12°C; ligations involving sticky ends, 4 hours at room temperature. The ligation mix was then diluted with an equal volume of T.E. and an aliquot used for transformation. Any unused reaction mix was stored at -20°C until required.

2.8.12 Transformation of *E.coli* cells

Cells were made competant as described earlier. To a 200µl aliquot was added 10-200ng of DNA in a volume not exceeding 20µl. The cells were left on ice for 30 minutes, then heat shocked at 42°C for 90 seconds. 0.8ml of L-Broth was added and the cells were left at 37°C for 45 minutes, or 30°C for 60 minutes to allow expression of the antibiotic resistance. 0.2ml of a 10^0 and 10^{-1} dilution were plated on the appropriate selective plate and the plates incubated at either 37°C or 30°C in the case of temperature sensitive strains.

TABLE 2.8 Solutions for Agarose Gel Electrophoresis

50x Tris/acetate/EDTA pH 8.2 electrophoresis buffer:

2.0M Tris base (Tris[hydroxymethyl]aminomethane)
1.0M sodium acetate
0.05M EDTA (ethylenediaminetetraacetic acid)

Dissolve 605.5g Tris base, 340g sodium acetate ($CH_3COONa.3H_2O$), and 46.5g EDTA (bisodium salt) in 1.5l of distilled H_2O and adjust to pH 8.2 with glacial acetic acid. Make up to a final volume of 2.5l, check the pH again, and filter the solution through a fluted circle of Whatmann No. 50 paper. Store the filtered solution in a 2.5l brown glass Winchester bottle at room temperature.

5X Tris/acetate/EDTA loading buffer:

Working Concentration of Constituents		5x Buffer
1x	electrophoresis buffer	5x
3.0%	Ficoll 400	15.0%
50mM	EDTA	0.25M
0.04%	bromophenol blue	0.2%
0.04%	xylene cyanol FF	0.2%

Dissolve 40mg of both xylene cyanol FF and bromophenol blue in 10ml of 0.5M EDTA solution (pH 8.0), add 2ml of 50x Tris/acetate/EDTA electrophoresis buffer stock solution, then add 3g of solid Ficoll 400, mix thoroughly until fully dissolved, and make up to a final volume of 20ml with distilled H_2O. Dispense 2.5ml aliquots into 5ml glass Bijou bottles, label, and store at 4°C.

2.8.13 Agarose gel electrophoresis of DNA

For rapid analysis of DNA, BRL Horizontal Gel Apparatus Model H6 (Minigel) was used. For more precise measurement of restriction fragments, and where preparation of specific DNA fragments was desired, BRL Apparatus H5 (Midigel) was used. Depending on the fragment size range under analysis, gels of between 0.7 - 1.5% agarose were used (Sigma Type 1, low EEO). The required amount of agarose was added to 1x Tris Acetate Electrophoresis Buffer (table 2.8), the bottle weighed, and the agarose melted by heating in a microwave oven. The bottle was again weighed and distilled H_2O was added to make up for any evaporation incurred during heating. Approximately 10–15ml of molten agarose (cooled to 55°C) was used to pour a minigel, and 80–100ml for a midigel. After the gel had set, the comb was removed and the tank filled with buffer. The DNA sample to be analysed was diluted with $^1/_5$ the volume of loading buffer (table 2.8), and loaded into a well which had been pre-filled with running buffer. Well volumes of 5–10µl and 10–15µl for minigels and midigels respectively were commonly used. Minigels were run at 70V constant voltage for roughly one hour. Midigels were run at 20V constant voltage overnight. After the

run the gels were stained for 45 minutes in water to which ethidium bromide at 0.5µg ml^{-1} had been added. For very small amounts of DNA visualisation was sometimes improved by de-staining in water alone for 30 minutes prior to illumination of the DNA band under UV light. The gel was photographed using Ilford FP4 film and an exposure time of 8 seconds.

2.8.14 Radiolabelling of DNA fragments

The technique of Feinberg and Vogelstein (1983, 1984) was found to be most effective in the production of DNA probes labelled to a high specific activity. Either linearized plasmid DNA, or specific restriction fragments isolated from agarose gels using one of the previously described methods (section 1.7.6/7) was used as a source of DNA. The reaction was carried out at room temperature, and the constituents added in the following stated order: (i) H_2O to a total volume of 50µl; (ii) 10µl oligo-labelling buffer (see below). (iii) 2µl of BSA (10mg ml^{-1}); (vi) DNA in T.E. or dH_2O; (v) 5µl of ^{32}P dCTP at 5-10 µCi $µl^{-1}$ (Amersham International plc.); (vi) 2 units of Klenow fragment of *E.coli* DNA polymerase I. The reaction was allowed to proceed for 4 hours and was stopped by the addition of 200µl of STOP solution (see below). Purification of the precursor nucleotide triphosphates from the labelled product prior to hybridization was found to be unnecessary. Oligo labelling buffer:- A:B:C in the ratio 2:5:3 (v:v): A= 1.25M Tris-HCl pH 8.0, 0.125M $MgCl_2$, 0.5mM each of dATP, dTTP, dGTP, and 0.25mM 2-mercaptoethanol; B= 2M Hepes-NaOH pH 6.6; C= 4µg $µl^{-1}$ random hexadeoxynucleotide (pd(N)6) mixture in T.E. STOP solution:- 20mM NaCl, 20mM Tris-HCl pH 7.5, 2mM EDTA, 0.25% (w:v) SDS, 1mM dCTP.

2.8.15 Southern hybridisations

For the detection of specific DNA fragments the widely used technique of transferring DNA fragments to nitrocellulose sheets followed by hybrisation with a radiolabelled probe was used (Southern, 1975)

Transfer of DNA to nitrocellulose filters: DNA was separated on agarose gels as previously described. The DNA was denatured by soaking the gel in 0.5M NaOH, 1.5M NaCl at 37°C for 1 hour, followed by neutralisation in 1.0M CH_3COONH_4, 0.02M NaOH for 1 hour, again at 37°C. The gel was placed on a glass sheet and overlaid with a sheet of nitrocellulose (Schleicher and Schuell, 0.45µm pore size, soaked in 2 x SSC) cut 3mm larger than the gel. Two sheets of Whatmann 3MM paper were placed over this, followed by a 2 inch stack of paper towels. A 1kg weight was placed atop and the transfer allowed to proceed overnight. The filter was washed in 2 x SSC, dryed at

37°C, before being baked under vacuum at 80°C for 2 hours.

Hybridisations: Hybridisations were carried out in heat-sealed polythene bags in as small a volume as possible. The solutions used are listed below.

(Pre-)hybridisation fluid

20x SSC	1ml
Formamide	5ml
50x Denhardts solution	0.2ml
Salmon Testes DNA (10mg ml^{-1})	0.25ml
10% SDS	0.5ml
H_2O[1]	3.05ml
	10ml total

[1] The volume of the probe was subtracted from this volume when performing the actual hybridisation.

20x SSC consists of 3.0M NaCl, 0.3M sodium citrate (877g of NaCl + 441g of tri-sodium citrate [$Na_3C_6H_5O_7.2H_2O$] in 4l of dH_2O, adjusted to pH 7.0 with NaOH, made up to 5l final volume.

50x Denhardts solution contains 1% (w:v) bovine serum albumin (Sigma; Fraction V, 98-99%); 1% (w:v) Ficoll (Sigma; Type 400); 1% (w:v) polyvinylpyrrolidone, 0.45µm Millipore filtered.

Filters were placed in polythene bags, the pre-hybridisation fluid was added, the bag sealed, and the filters were shaken at 37°C for 2 hours. The probe was added to a second batch of pre-hybridisation fluid in a 25ml Macartney bottle and this was placed in a boiling water bath for 10 minutes to denature the probe. The pre-hybridisation bag was cut open, the fluid discarded, and the hybridisation mix was added (pre-hybridisation fluid plus the probe). The filters were returned to 37°C and left to hybridise overnight. The filters were washed at 37°C using the following schedule;

- 2x 30 minutes in 50% (v:v) formamide, 2xSSC, 0.5% SDS (20-30ml)
- 4x 30 minutes in 2x SSC, 0.5% SDS (100-200ml)
- 2x 5 minutes in 2xSSC (100-200ml)

Filters were allowed to dry at 37°C. If autoradiography was required the filter(s) were wrapped in parafilm and exposed to Cronex 4 X-ray film at -70°C in a

Cronex autoradiography cassette fitted with a phosphotungstate intensifier screen.

2.8.16 Determination of plasmid copy number by quantitative hybridisation

A fuller discussion of this technique is presented in Chapter 8. The version shown below was found to yield the most reproducible results for ColE1 type replicons.

Cells were grown in L-Broth + antibiotic until steady state growth was achieved. This involved making several dilutions into pre-warmed media such that the growth rate was maintained between $O.D._{540}$ of 0.1-0.2. 6ml samples were taken (simultaneously measuring the $O.D._{540}$), chilled on ice, and pelleted in a bench top centriguge at 4°C. The pellet was resuspended in 0.6ml of ice-cold T.E. and sonicated for 3x 10 seconds using an MSE 100w Ultrasonic Disintigrater and titanium probe at 6 µm peak to peak amplitude. The cultures were maintained in an ice bath during this step. 4ml of 15x Basic SSPE (100ml of 20x SSPE pH 7.0 and 30ml of 2M NaOH) was added and the extract transferred to 80°C for 10 minutes. 20x SSPE is (per litre) 174g NaCl, 27.6g $NaH_2PO_4.H_2O$, and 7.4g of EDTA adjusted to pH 7.4 with 10N NaOH. The lysate was neutralised by the addition of 1.2ml of acidic Tris-HCl (100ml of 2M Tris and 21ml of concentrated HCl) and placed on ice. It was found that the extract could be stored at -20°C until required if desired. 100, 250, 500, 750 and 1000µl extracts were spotted onto nitrocellulose filters under a slight vacuum. Filters were prepared by cutting 1.5mm squares from a sheet of 0.45µM pore size nitrocellulose paper (Schleicher and Schuell) and soaking in distilled H_2O for 10 minutes at 70°C, prior to transfer to a solution of 10x SSPE where they were kept until use. Pencil was used to mark the filters to allow identification. After the extract had been spotted onto the filters they were dried at 37°C prior to baking at 80°C for two hours. 100ng of purified plasmid DNA (usually pBR325) was used as the probe DNA, with hybridisations being carried out in a volume of 10ml. The filters were pre-hybridised, hybridised, and washed as described above. Dried filters were transferred to scintillation vials containing 0.4% Butyl-PBD in toluene and the radioactivity counted. Counts were plotted against extract volume to give a straight line, the gradient of which could be used to estimate the copy number relative to the Optical Density at the time of sampling.

2.8.17 Recovery of plasmid DNA from sonicated cell extracts

Cells were grown in L-Broth with selection until an $O.D._{540}$ of 0.2 was reached. 5ml of cells were transferred to a sterile Bijous bottle and incubated on ice for 5

minutes. The cells were pelleted by centrifugation at 4°C for 5 minutes, then resuspended in 0.3ml of T.E. The sample was sonicated for the required period (1-60 seconds), being kept on ice throughout. The lysate was then transferred to a 1.5ml eppendorf tube and the debris removed by centrifugation at 4°C. The supernatant (280µl) was transferred to a fresh eppendorf, 1ml of ethanol added, kept on ice for 5 minutes, then centrifuged at 4°C for 10 minutes. The supernatant was then discarded. If the object was merely to visualise the plasmid on a gel, the precipitate was resuspended in 20µl of T.E. and loaded directly onto an agarose gel. If Southern Transfer was required (section 2.8.15), de-proteination was found to be necessary for efficient transfer of the DNA from the gel. In this case the pellet was resuspended in 200µl of ice-cold T.E., 100µl of ice-cold 7.5M NH_4 Acetate was added, and the eppendorf left on ice for 20 minutes, followed by centrifugation for 6 minutes at 4°C. The supernatant was transferred to a new eppendorf, 1ml of ethanol added, kept on ice for 5 minutes, then centrifuged at 4°C for 10 minutes. The supernatant was discarded and the pellet resuspended in 10µl of T.E. prior to loading on an agarose gel.

2.9 Protein techniques

2.9.1 ^{35}S-methionine labelling of proteins in minicells

An aliquot of purified minicells was allowed to thaw at room temperature. 1ml of M9 glucose medium was added, the suspension was mixed by vortexing, and the minicells were pelleted by spinning in a microfuge for 2 minutes. The pellets were resupended in 100µl of M9 glucose medium, and the label (usually 2.5µl) was added in Difco Methionine Assay Mix to a total volume of 10µl. The minicells were incubated at 37°C for 15 minutes, the reaction being stopped by the addition of 900µl ice-cold methionine chase and transfer to an ice bath. This was followed by centrifugation for 2 minutes in a microfuge at 4°C. The pellet was resuspended in 1ml ice-cold 0.05M Tris-HCl pH 6.8, and centrifuged as before to remove unincorporated label. The pellet was resuspended in 25µl of loading buffer (table 2.9), boiled for 3 minutes, and loaded onto an SDS-polyacrylamide gradient gel.

2.9.2 ^{35}S-methionine labelling of proteins following Phage λ infection of UV-irradiated cells

A 5ml overnight culture in L-broth was set up from a single colony of the bacterial strain 159; 0.05mls of this being used to innoculate 10mls of M9 maltose medium at 37°C. The culture was grown until an $O.D._{540}$ of about 0.2 was reached. It was then transferred to a sterile Universal bottle, placed on ice for 5 minutes,

centrifuged at 4500 rpm for 5 minutes at 4°C, and the supernatant discarded. The cells were then resuspended in 1ml of ice-cold 1x M9 salts to which $MgSO_4$ had been added to 20mM. The suspension was estimated to contain about 10^9 cells per ml. It was then irradiated on ice for 7.5 minutes at 600 ergs mm^{-2} min^{-1}. Exposure to visible light was minimised to reduce phoreactivation. The culture was transferred to a foil wrapped eppendorf and kept on ice for 10 minutes to allow dissipation of free radicals and decay of pre-formed mRNA. 100µl aliquots were added to foil-wrapped eppendorf tubes already containing 2 x 10^8 phage (usually 10-100µl) and left for 10 minutes on ice. To each tube was added 2.5µl of ^{35}S-methionine diluted to 10µl in Difco Methionine Assay Mix. The cultures were incubated at 37°C for 15 minutes, prior to transfer to an ice bath for 5 minutes. The cells were centrifuged at 4°C for 2 minutes in a microfuge, the supernatant discarded, and resuspended in 1 ml of ice cold Tris-HCl pH 6.8 followed by a second centrifugation as before to remove unincorporated label. The pellet was resuspended in 25µl of loading buffer (table 2.9) prior to boiling and loading on a SDS polyacyrlamide gel as for minicells.

2.9.3 ^{35}S-methionine labelling of proteins using an *in vitro* prokaryotic DNA directed translation system

The system used was a modification of that described by De Vries and Zubay (1967), employing a Prokaryotic DNA Directed Translation Kit (Amersham International plc, code N.380), requiring only the addition of a purified DNA template. The protocol was essentially that described by the manufacturers.

The DNA template was prepared by caesium chloride equilibrium density centrifugation and was added at a concentration of 0.5µg μl^{-1} in T.E. buffer, either as intact plasmid DNA or following digestion by restriction endonucleases. DNA was restricted as previously described (section 2.8.8), followed by ethanol precipitation (section 2.8.5) to remove proteins and salt. Incorporation of ^{35}S-methionine was found to be most efficient with covalently closed circular DNA; linearised plasmid DNA only resulting in about 10% incorporation in comparison.

The kit was stored at -70°C until use. The contents were then thawed slowly on ice and the reaction set up as follows in a 1.5ml microfuge tube:-

Standard DNA	2.5µl
Supplement Solution	3.8µl
Amino acids minus methionine	1.5µl
^{35}S-methionine (20µC µl^{-1})	1.0µl
Dilution buffer	3.7µl
S-30 Extract	2.5µl
	15.0µl Total

A DNA free control was included in each experiment; in this instance 6.2µl of dilution buffer was added to bring the volume up to 15µl.

The contents were mixed gently and the tube incubated at 37°C for 1 hour. 2.5µl of methionine chase solution was then added, and incubation was allowed to proceed for a further 5 minutes at 37°C. The reaction was terminated by placing the tubes into an ice bath. 15µl of loading buffer (table 2.9) was added and the tubes were boiled for 3 minutes, prior to storage until required at -20°C or else immediate loading (10-30µl) onto an SDS-polyacrylamide gel followed by electrophoresis (section 1.8.4).

2.9.4 SDS-polyacrylamide gel electrophoresis

The gel system was essentially that of Laemmli (1970), and depending on the application, either gradient gels of 14% - 20% or linear gels were used. Equipment used was Hoefer Scientific Instruments Slab Gel Units nos. SE 250 (minigels) or SE 600 (full size).

The solutions used are shown in table 2.9. Equipment was assembled as per manufacturers instructions, spacers of 0.75mm being used between the plates of the glass sandwich. For rapid screening of protein products etc., mini protein gels of between 8-14% acrylamide were used, their design allowing completion of a run in under an hour. For preparative runs, or where greater separation of proteins was required, full size gel equipment was used, either as an 8% linear gel, or a 14-20% gradient gel. The composition of typical gel solution mixtures is shown on the following page. The ratios of the solutions were varied depending on the required acrylamide concentration.

For 10ml separating gel solutions:-

	8%	14%	20%
50% Glycerol	0.1ml	0.15ml	0.7ml
Acrylamide Stock	2.66ml	4.65ml	6.65ml
4x Separating Gel Buffer	2.5ml	2.5ml	2.5ml
dH_2O	4.5ml	2.5ml	----

At this stage the solution was de-gassed under vacuum

10% SDS	0.1ml	0.1ml	0.1ml
AMPS[1]	0.075ml	0.075ml	0.075ml
TEMED[2]	4µl	1µl	0.5µl

For 10ml stacking gel solution (4% acrylamide):-

Acrylamide Stock	1.33ml
4x Stacking Gel Buffer	2.5ml
dH_2O	6.0ml

At this stage the solution was de-gassed under vacuum

10% SDS	0.1ml
AMPS[1]	0.075ml
TEMED[2]	7.5µl

[1] 10% Ammonium Persulphate solution made immediately prior to use.
[2] N,N,N',N'-Tetramethylethylenediamine.

3-4ml of gel solution was required to pour a minigel; 16ml being required for a full size gel. For linear gels the gel mixture was poured directly between the glass plates using a pipette. For gradient gels the following procedure was adopted. To slow down polymerisation the solutions were chilled on ice prior to addition of the TEMED. After addition, 8.5ml of the 20% solution was added to the outer chamber of a gradient maker, the connection channel opened, and a small amount allowed to flow through to the other chamber (to remove any air bubbles). The connection channel was closed and 8mls of the 14% solution was poured into the empty chamber. A magnetic flea was used to stir the solution in the outer chamber. A clamp on the outlet tube was opened and approximately 0.5ml of the 20% solution was allowed to flow out. The clamp was then closed, the connecting channel opened, the clamp re-opened, and the gel solution allowed to flow down the tube and between the glass plates at a flow rate of 2ml min^{-1}. After the separating gel had been poured (linear or gradient gel), it was carefully overlaid with iso-butanol saturated with 1x separating gel buffer and left to polymerise for 1 hour. This was indicated by the appearance of a sharp interface at the upper surface of the gel. The iso-butanol was discarded and the top of the gel

TABLE 2.9 Solutions used in SDS-polyacrylamide gel electrophoresis

- **Stock Acrylamide**: 30g acrylamide, 0.8g NN'Methylene bis-acrylamide. Made up to 100ml with distilled H_2O, 0.22µM Millipore filtered and stored in the fridge.

- **4x Stacking gel buffer (0.5M Tris)**: 15.25g of Tris base, dissolved in 200ml distilled H_2O, adjusted to pH 6.8 with concentrated HCl, made up to 250ml and filtered as above.

- **4x Separating gel buffer (1.5M Tris)**: 45.5g of Tris base, dissolved in 200ml distilled H_2O, adjusted to pH 8.8 with concentrated HCl, made up to 250mls and filtered as above.

- **10x Reservoir Buffer**: 30.2g of Tris base, 144g of glycine dissolved in distilled H_2O to 1l, filtered as above. SDS was added to 10% in the final 1x buffer.

Loading buffer	4 x Stacking Gel Buffer	0.125ml
	10% SDS	0.300ml
	50% Glycerol	0.200ml
	2-mercaptoethanol	0.050ml
	0.1% Bromophenol Blue	0.200ml
	dH_2O	0.125ml
	Total Volume	**1.0 ml**

was washed several times with distilled H_2O, followed by once with the stacking gel solution to which the TEMED had been added. This was quickly poured off, the gel sandwich re-filled, and a 0.75mm Teflon comb inserted. After polymerisation, the comb was carefully removed and the wells washed with distilled H_2O. The gel apparatus was assembled and 1x Reservoir Buffer poured into the upper and lower tanks. Minigels required about 200ml of buffer; full size gels required about 4.5 litres. Samples and molecular wieght standards were loaded into the wells using a 50µl Hamilton Syringe. About 8µl of sample could be loaded onto a minigel; this volume being 40-50µl for the full size gel, although protein concentration in the sample was found to be a more important determinant of sample size. Molecular wieght markers were either (a) Low Molecular Wieght Calibration Kit, Pharmacia Fine Chemicals for general use, and where pre-stained markers were required, (b) Rainbow^{TM} Markers from Amersham International plc. Gels were generally run until the bromophenol blue dye had just run off the end of the gel. For minigels this involved a constant current setting of 40mA and a run length of about 50 minutes. Full size gels were generally

run at 10mA constant current overnight. Depending on the application these conditions could be varied; for example, preparative runs were generally run at a higher current setting and the progress monitored by observation of pre-stained markers. After a run, the current was switched off and the gel apparatus dis-assembled. The glass plates were gently prised apart with a plastic spatula and the gel transferred to a tupperware container for staining. Staining and destaining procedures were carried out at 37°C. Gels were stained for 60 minutes in a solution of 9% (v:v) CH_3COOH, 45% (v:v) CH_3OH, and 0.1% (w:v) Coomassie Blue. They were then destained for 2 x 30 minutes in a solution of 5% (v:v) CH_3OH, 7% (v:v) CH_3COOH. In preparative SDS Gel Electrophoresis the required protein band was cut out of the gel at this stage prior to electroelution (section 2.9.6). Otherwise the gels were soaked for 30 minutes in a solution of 2% (v:v) glycerol, 10% (v:v) CH_3OH, transferred onto a sheet of Whatmann 3MM paper and dried down under vacuum, 80°C for 45 minutes. If required, autoradiography was performed using Cronex 4 X-ray film in a Cronex autoradiography cassette at room temperature.

2.9.5 Induction of proteins under control of P*gal*

When induction of proteins under control of the galactose operon promoter was required, the following procedure was adopted. This was found to give significantly better induction than a simple addition of IPTG.

A single colony was used to innoculate 10ml of L-Broth supplemented with 0.2% glucose and antibiotic selection where appropriate. The culture was grown at 37°C until an $O.D._{540}$ of c. 0.8 was reached, corresponding to late log phase growth. The cells were pelleted in a bench top centrifuge (at room temperature) for 5 minutes at 4500rpm, then resuspended in 25ml of the following media:-

1x VB salts (table 2.4)
5mg ml^{-1} Casamino acids (table 2.5)
1mM cAMP
20µg ml^{-1} IPTG

Additional nutritional requirements
to the concentrations shown in table 2.5

The culture was returned to 37°C and allowed to grow for 2 hours. The cells were then chilled on ice, pelleted, washed in bacterial buffer (table 2.4), pelleted again, and finally stored at -20°C until required.

2.9.6 Purification of β-galactosidase fusion proteins by affinity chromatography

The protocol followed was essentially that of Ullman (1984). For a 10ml column, 2.5g of CH Sepharose 4B (Pharmacia) was transferred to a 25ml universal bottle and suspended in excess 0.5ml NaCl. It was then poured onto a scintered glass column (Millipore) and washed with 500ml of 0.5M NaCl to remove any additives, followed by 100ml of distiled H_2O (pH ajusted to 4.5). The resin was transferred to a 100ml glass beaker and 28.7mg of TPEG (p-aminophenyl-β-D-thiogalactoside; Sigma) in 1ml of distilled H_2O was added. The pH of the resin was adjusted to 4.7, and distilled H_2O was added to give a final gel concentration of 2:1 (liquid:gel). To this slurry was added, dropwise, 144mg of N-ethyl-N'(3-dimethylaminopropyl)-carbodiimide (Sigma) in 8 ml of distilled H_2O. The slurry was left to shake gently at room temperature for 1 hour while the pH was maintained at 4.7. After this period, no detectable changes in pH were observed, and the reaction was allowed to proceed for 24 hours at room temperature with gentle shaking. The resin was then used to pack a 20cm Pharmacia C16 column with AC16 adaptor as per manufacturers instructions. The column was washed with 500ml of 1M NaCl followed by 500ml of distilled H_2O and finally 500ml of running buffer. The column was stored and run at 4°C. Running buffer is:- 20mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 1.6M NaCl, 10mM β-mercaptoethanol.

To run the column, induced cells (section 2.9.5) were washed and concentrated 20 fold in 20mM Tris-HCl pH 7.4, 10mM $MgCl_2$, lysed by sonication in an MSE sonicator (6μM peak to peak), then cleared by centrifugation (10 000 rpm for 30 minutes). The supernatant was diluted 1:10 into running buffer and slowly loaded onto the column (flow rate of 1ml cm^2 min^{-1}). The column was then washed with 4 litres of running buffer at a flow rate of 10ml cm^2 min^{-1}. The fusion protein was eluted in 0.1M NaBorate pH 10.0, 10mM NaCl. Aliquots of 1ml were taken and tested for β-galactosidase activity; 90% of the activity eluted in a volume of 3-4 ml. The fusion protein eluate was then neutralised by the addition of an equal volume of 0.1M Na Phosphate pH 6.0, prior to storage at -20°C until required.

2.9.7 Electroelution of proteins from SDS-polyacrylamide gels

Gels were run and stained as previously described (section 2.9.4). The desired protein band was cut out using a razor blade and transferred to the elution chamber of the Biotrap apparatus . The apparatus was set up as for electroelution of DNA fragments from agarose gels (section 2.8.16), except reservoir buffer (table 2.9) was added to the tank and elution chamber. The power pack was set on a constant voltage setting of 200v, and the electroelution allowed to proceed for 20 hours at 4°C.

An aliquot of the eluted protein solution was run on an SDS-polyacrylamide gel to check the recovery efficiency; the remainder was stored at -20°C until required.

2.10 Immunological techniques

2.10.1 Preparation of antigen

The fusion protein was purified by SDS-polyacrylamide gel electrophoresis (section 2.9.4), followed by electroelution from the gel slice into reservoir buffer (section 2.9.6). The purified protein was usually obtained in a volume of 4-500µl, containing roughly 0.1-1.0mg of protein. An aliquot of this was run on an SDS-polyacylamide gel as a test of purity and yield. If satisfactory, the preparation was emulsified with 2 volumes of Freunds Incomplete Adjuvant (Sigma) prior to injection. After mixing with the adjuvant the antigen preparation was injected within a few hours. Freunds Incomplete Adjuvant was used throughout the immunisation procedure in preference to Complete Adjuvant in an attempt to reduce the risk of cross-reactivity due to the presence of mycobacterial proteins in the latter formulation.

2.10.2 Immunisation and bleeding schedules

Three rabbits were used for antibody production so that a choice of antisera would be available for experimental work. Immunization, bleeding, and general maintainance of the rabbits was carried out at the Animal Faculty Area, Dept. of Biochemistry, University of Edinburgh.

Each rabbit was injected with about 0.5ml of antigen/adjuvant, split between 2 subcutaneous injection sites (i.e 0.25ml per injection site). Animals were bled prior to the first injection to obtain pre-immune sera (4ml). Thereafter rabbits were injected monthly over the next four months (i.e five injections in all). Ten days after the final injection the rabbits were bled of 15ml of blood and the sera tested for anti-DnaA activity. As this proved satisfactory no further bleeding of the rabbits was performed.

2.10.3 Antiserum processing

. Following bleeding of the rabbit the blood was left at 4°C overnight while a clot was allowed to form. The serum was cleared by centrifugation at 13 000g for 10 minutes followed by addition of NaN_3 to a final concentration of 0.02% prior to storage at -20°C until required.

To find the best dilution of antisera for use in Immunogold Silver staining of protein blots (section 2.10.5), dilutions of 1:100, 1:500, 1:1000 and 1:2000 were tested. The dilution which gave the strongest signal with the lowest background was used in all subsequent experiments. In addition, this allowed choice of the best antiserum from the three rabbit sera available.

2.10.4 Western Blotting of Proteins

Electrophoretic transfer of proteins from SDS-polyacrylamide gels onto nitrocellulose paper (Western Transfer) was carried out as follows, using a Bio Rad Trans-Blot Cell (model 1703910).

Protein samples were run on SDS-polyacrylamide gels as described previously (section 2.9.4). Pre-stained markers (Rainbow Markers, Amersham) were included on each gel. The transfer sandwich was set up as follows:- 2 layers of foam spacers; 3 layers of Whatmann 3MM Paper, 0.5cm larger than the gel; nitrocellulose paper (Schleicher & Schuell, 0.45μM) the same size as the gel; the gel; nitrocellulose paper, same size as gel; 3 layers of Whatmann 3MM Paper, 0.5cm larger than the gel; 2 layers of foam spacers. The second nitrocellulose filter distal to the positive electrode was included to cut down background due to any extraneous proteins present in the buffer/equipment etc. Each layer was wetted in transfer buffer before assembly of the sandwich, and air bubbles and excess buffer between each layer were carefully removed by rolling a glass pipette over the surface. Transfer buffer is; 0.25M Tris, 1.92M Glycine, 20% v/v methanol, 0.1% w/v SDS. The plastic folder containing the transfer sandwich was closed and checked to ensure that each layer was held tightly in place. It was then placed in the transfer apparatus and covered with buffer. Transfer was accomplished in 3 hours at a setting of 70 volts with water cooling. After transfer the sandwich was dissasembled and the nitrocelluose filter was rinsed once in transfer buffer without SDS prior to drying between filter paper. The membrane was then wrapped in parafilm and stored at 4°C until required. The gel was then stained as previously described (section 2.9.4) to check the efficiency of transfer.

2.10.5 ImmunoGold Silver Staining of protein blots

AuroProbe^{TM} BLplus reagents (Janssen Biotech N.V.) were used as means of visualising specific protein bands after Western Transfer. The non-occupied protein binding sites were first saturated by incubating the transfer membrane in blocking solution at 37°C for 40 minutes. The volume required depended on the membrane

size, but was usually in the region of 10-20 ml. Blocking was carried out in a sealed plastic bag. 1x blocking solution is, per 250ml; 12.5g BSA Sigma No. A-7906 (5% final w/v), 2.5ml of 2.0M Tris-HCl pH 8.2 (20mM), 2.25g NaCl (0.9% final w/v), 0.325g Na Azide (20mM). The membrane was then washed three times for 5 minutes in 1x washing solution. This, and all subsequent steps were performed at room temperature. 10x washing solution is, per 250ml; 2.5g BSA Sigma No. A-7906 (1% final w/v), 25ml of 2.0M Tris-HCl pH 8.2 (200mM), 22.5g NaCl (9% final w/v), 3.25g Na Azide (200mM). The saturated transfer membrane was then incubated, under constant agitation, for 2 hours in a sealed plastic bag with the primary antibody (anti-DnaA). A suitable dilution of the primary antibody together with a final concentration of 1% goat normal serum in 1x blocking solution was used. The manufacturers recommend that binding of the primary antibody be performed in 1x washing solution; however, use of 1x blocking solution was found to result in less background staining of the filter. A volume of 10-20ml was generally used for this step. The membrane was then washed as before in 1x washing solution before incubating for 2 hours with the secondary antibody, using the same type of incubation vessel and volume as for the initial incubation. The solution used for this incubation was a 1:100 dilution of AuroProbe^{TM} BLplus (secondary antibody) into 1x blocking solution supplemented with a 1:20 v/v final dilution of gelatin. The gelatin used was supplied with the kit. The membrane was subsequently washed for 3x 5 minutes in washing solution, then 2x 1 minute in distilled water. The image was intensified by incubating the membrane in a 1:1 mixture of Initiator and Enhancer solutions from the IntenSE^{TM} silver enhancement kit (Janssen Biotech N.V.) Enhancement was monitored visually and interrupted after 10-15 minutes. If a stronger signal was desired, the membrane was further incubated in a fresh mixture of Initiator and Enhancer solutions; if left for more than 15-20 minutes in the intensifying solution self-nucleation, and hence background, became a problem. Finally, the membrane was washed in several changes of distilled water after which it was dried between filter paper.

CHAPTER 3

Sub-cloning of the *groE* genes and an investigation of GroEL protein homologies

3.1 Introduction

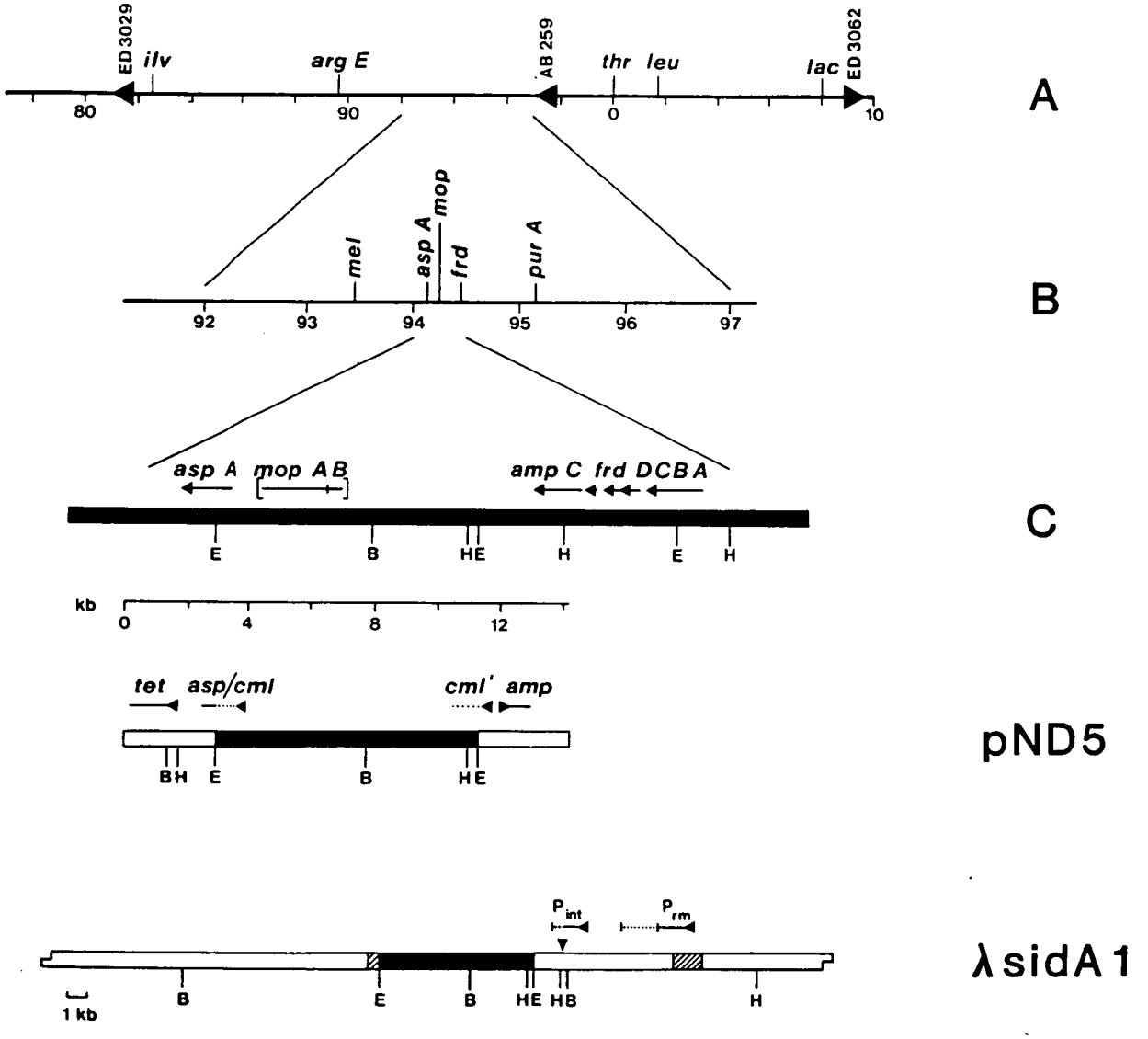
As has already been discussed in chapter 1, the *groE* genes were originally identified as a high copy number suppressor of *dnaA* during an attempt to clone the *dnaA* gene into the λ replacement vector λNM616 (Mileham *et al.*, 1980). The resultant phage, λ *sidA* (Jenkins, 1985) carried an 8.1kb *Eco*RI insert, which appeared to be able to mediate suppresion as a result of its new molecular environment. The actual suppressor itself was termed *sidA*. Suppression appeared to result from an increased copy number of this DNA fragment; suppression by λ *sidA* was a *recA* dependant event, involving multiple lysogenisation of the phage (Jenkins, 1985; Fayet *et al*, 1986). Similarly, *sidA* was able to suppress when cloned into a high copy number vector, in this instance the *Eco*RI site of pBR325 (Jenkins, 1985). This construct was called pND5, and is shown, together with λ *sidA* in figure 3.1.

That suppression was a result of over-expression of the *groE* genes was suggested by two observations. Firstly, mapping data and complementation of *groE* mutants demonstrated that the 8.1kb insert almost certainly contained the *groE* genes (Jenkins, 1985; Jenkins *et al*, 1986). Secondly, comparison of *sidA* with a supressor of *dnaA* reported by Takeda and Hirota (1982) revealed an identical restriction map. This suppressor had also been identified as a high-copy number suppressor of *dnaA*. They did not map their suppressor (which they termed *sdaA*), but provided good evidence that a protein which they measured at 68kd molecular weight was required for this suppression to occur. On protein gels in this laboratory the large protein encoded by the 8.1kb insert appeared to have a molecular weight of about 60kd, but since this is the only protein of about this size encoded by the insert it seemed likely that these two proteins were the same. As this size is similar to that reported for the GroEL protein (Georgopoulos and Hohn, 1978; Hendrix and Tsui, 1978), it seemed likely that suppression by *sidA/sdaA* (hereafter referred to as *sdaA*) was mediated, at least in part, by over-expression of *groEL*.

3.2 Construction of plasmids pJM18 and pJM32

Further characterisation of the suppressing insert was deemed neccessary before it could be unequivocally demonstrated that GroEL was indeed the gene product responsible. Furthermore, other proteins encoded by the insert might be required in addition to GroEL for suppression to occur. For this reason it was considered desirable to subclone fragments from the 8.1kb insert and delimit suppression to specific gene products. Plasmid pSR1 was constructed in this

FIGURE 3.1 Chromosomal location of *groE*: construction of pND5 and λ *sidA*



Genetic and physical map of the groESL (mopAB) region of the E.coli chromosome. A and B show location with respect to adjacent markers, with map distances marked in minutes. Physical map of the region is shown in C, with plasmid pND5 shown to the same scale. Filled area represents insert DNA, unfilled area vector DNA. Taken from Jenkins et al. 1986.

laboratory for this purpose (J. H. Pringle), and is shown in figure 3.2. This plasmid was isolated by selecting for insertions of the transposon Tn1725 into pND5, and screening for plasmids still able to suppress *dnaAts* mutants. A feature of this transposon is that it carries an *Eco*RI site at either end; thus insertion introduces novel *Eco*RI sites

which may be useful in cloning, in addition to providing some information about the location of the gene of interest. Restriction map data (J. H. Pringle and figure 3.3) showed that in pSR1 the transposon is inserted about 1kb from the end of the 8.1kb insert (see figure 3.2). Since GroEL, being c.60kd in size would require at least 1.6kb of DNA to encode it, the gene must be located somewhere on the remaining 7.1kb of DNA at the right hand end of the insert. As the 8.1kb insert contains a unique *Bam*HI site (figure 3.2), it was decided to digest plasmid pSR1 with *Bam*HI and *Eco*RI, re-ligate the various fragments, and screen the resultant recombinants for plasmids containing the required inserts. That is, the 4.4kb *Bam*HI-*Eco*RI vector fragment together with either the 3.3kb or 3.7kb *Bam*HI-*Eco*RI insert fragments. These could then be screened for their ability to suppress and their protein products visualised.

To construct the plasmids, 10µg of pSR1 was digested with excess *Bam*HI and *Eco*RI, phenol extracted, and ethanol precipitated. The DNA was resuspended in T.E. buffer and an aliquot run on a gel to test that digestion had gone to completion. The ligation reaction was set up in a volume of 20µl at a DNA concentration of 30ng μl^{-1}. 5µl of the ligation mix was used to transform strain MM19, which is *dnaAts*. The transformation mix was plated at 30°C and 42°C with selection for ampicillin resistance. At 30°C, 600+ colonies were obtained, whilst at 42°C only a single transformant appeared. 99 colonies from the 30°C plate and the single 42°C transformant were tested for growth at 42°C and sensitivity to tetracycline and chloramphenicol. This latter test was to eliminate reconstituted vector (pBR325). Of the 100 colonies, 1 was chl^s, tet^s and able to grow at 42°C (that originally isolated at 42°C), while 49 were chl^s, tet^s but unable to grow at 42°C. 5 of the latter type and the single 42°C transformant were screened to identify what plasmids they contained. All five non-suppressors contained the 3.3kb *Bam*HI-*Eco*RI insert fragment, while the transformant isolated at 42°C yielded a plasmid containing the 3.7kb *Bam*HI-*Eco*RI fragment. The former plasmid was named pJM18 and the latter pJM32. Their restriction maps are shown in figure 3.2, and a photograph of the digested plasmids together with pND5 and pSR1 is shown in figure 3.3.

3.3 Analysis of the protein products encoded by pJM18 and pJM32

In order to determine which proteins were encoded by each insert fragment, plasmids pJM18 and pJM32 were transformed into the minicell producing strain DS410 (Dougan and Sherratt, 1977). Using about 50ng of DNA of each plasmid resulted in 6 x 10^3 transformants for plasmid pJM18, while only 3 transformants were obtained for

FIGURE 3.2 Restriction maps of pND5, pSR1, pJM18 and pND5

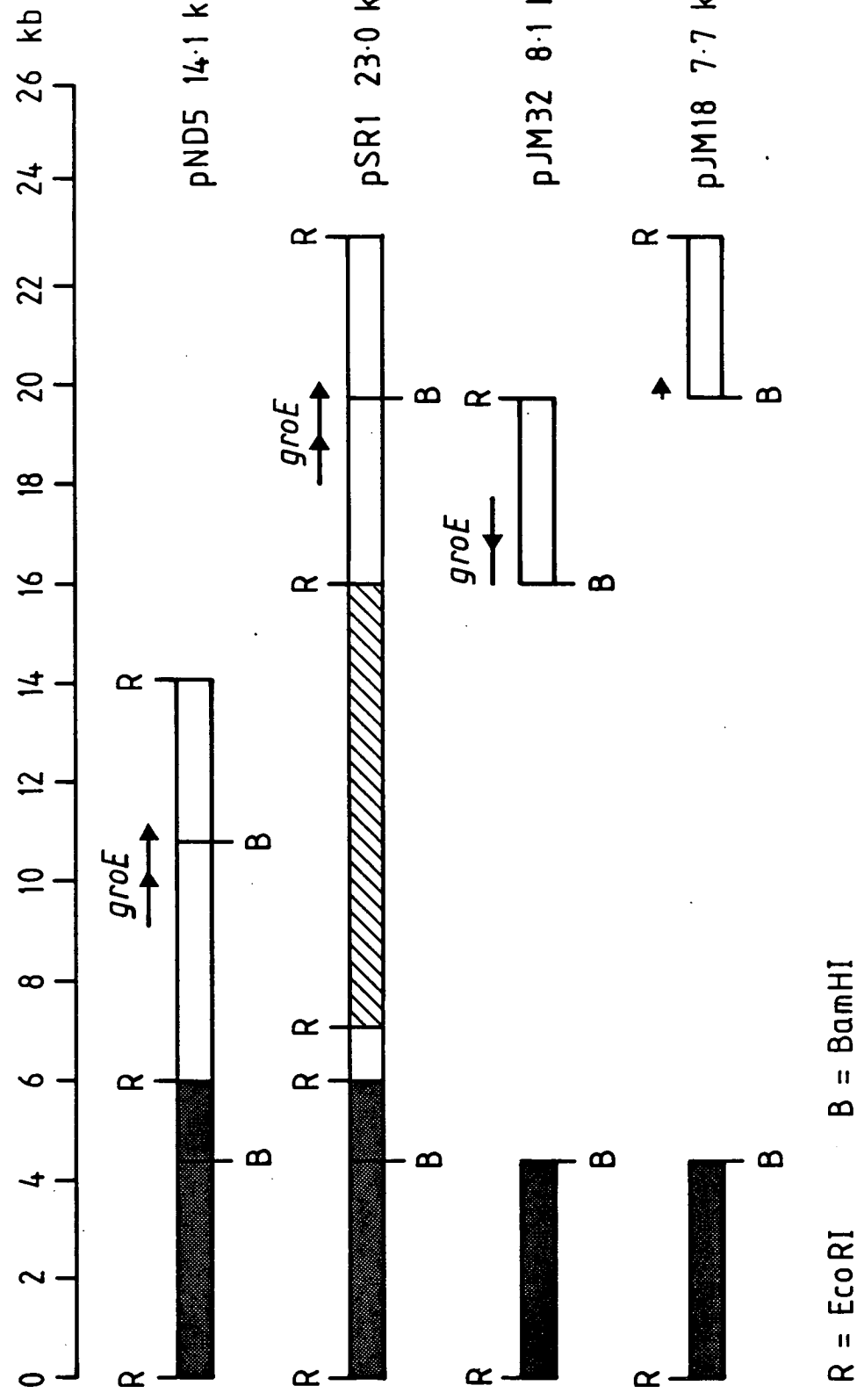
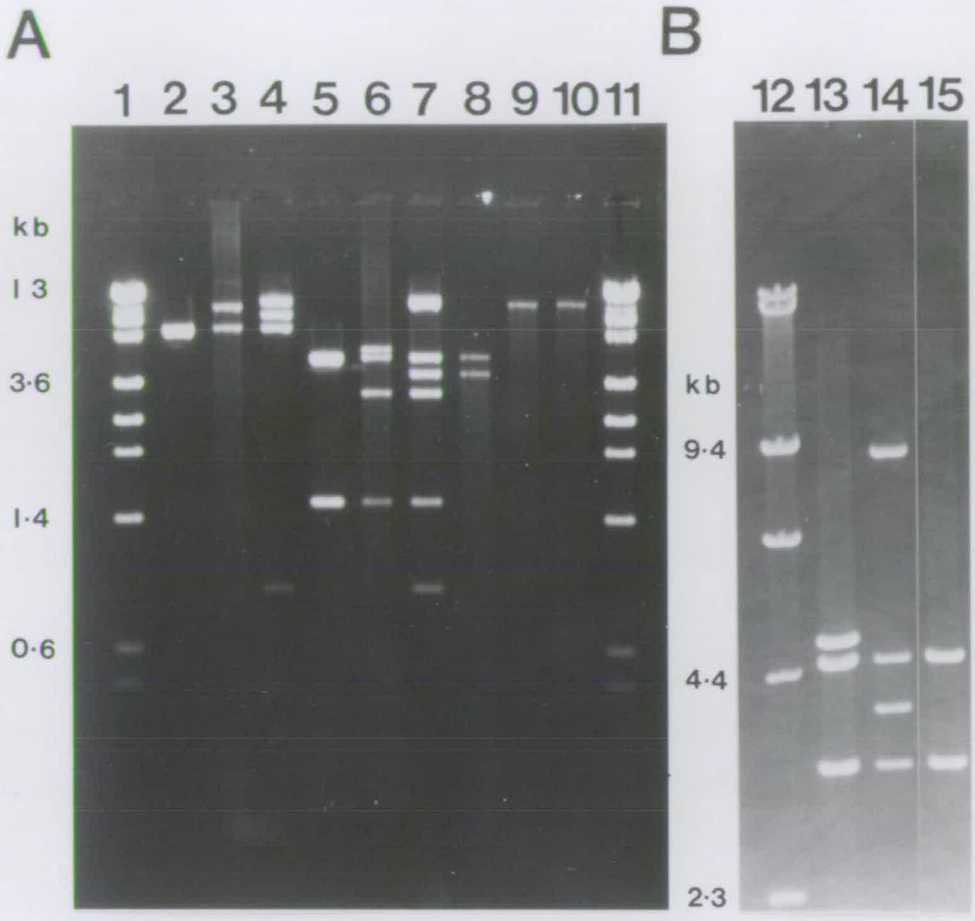


FIGURE 3.3 Restriction digests of pJM32, pJM18, pND5, and pSR1



A Gel number 1

Track 1: λcI^{857} AccI size standards
Track 2: pBR325 EcoRI
Track 3: pND5 EcoRI
Track 4: pSR1 EcoRI
Track 5: pBR325 EcoRI-BamHI
Track 6: pND5 EcoRI-BamHI
Track 7: pSR1 EcoRI-BamHI
Track 8: pJM32 EcoRI-BamHI
Track 9: pJM32 EcoRI
Track 10: pJM32 BamHI
Track 11: λcI^{857} AccI size standards

B Gel number 2

Track 12: λcI^{857} HindIII size standards
Track 13: pND5 EcoRI-BamHI
Track 14: pSR1 EcoRI-BamHI
Track 15: pJM18 EcoRI-BamHI

pJM32. Several transformants of each type were screened by DNA preparation and restriction to confirm the presence of the intact plasmid. As this proved satisfactory, radiolabelling of plasmid encoded proteins using ^{35}S-methionine in purified minicells was performed. The protein products were separated on an SDS-polyacrylamide gel and visualised by autoradiography. This is shown in figure 3.4. The GroES and GroEL produced by plasmid pND5 are very strongly expressed, and can be seen not only in the autoradiograph but also in stained minicells. Since pJM18 was unable to suppress *dnaAts* it was expected that at least *groEL* would be missing from this plasmid. This appears to be the case; while pJM18 encodes the 38kd and 32kd proteins of pND5, both GroE proteins are absent. However, the most suprising result came from plasmid pJM32. Instead of the expected GroE proteins, the autoradiograph for this plasmid revealed no plasmid encoded proteins, as though the plasmid were failing to segregate into the minicells. Examination of whole stained minicells revealed no strong GroE protein bands, in marked contrast to those seen with pND5. Although there appeared to be a slight increase in the amount of GroES and GroEL present in whole stained cells, this was neglible compared to that exhibited by pND5 containing whole cells.

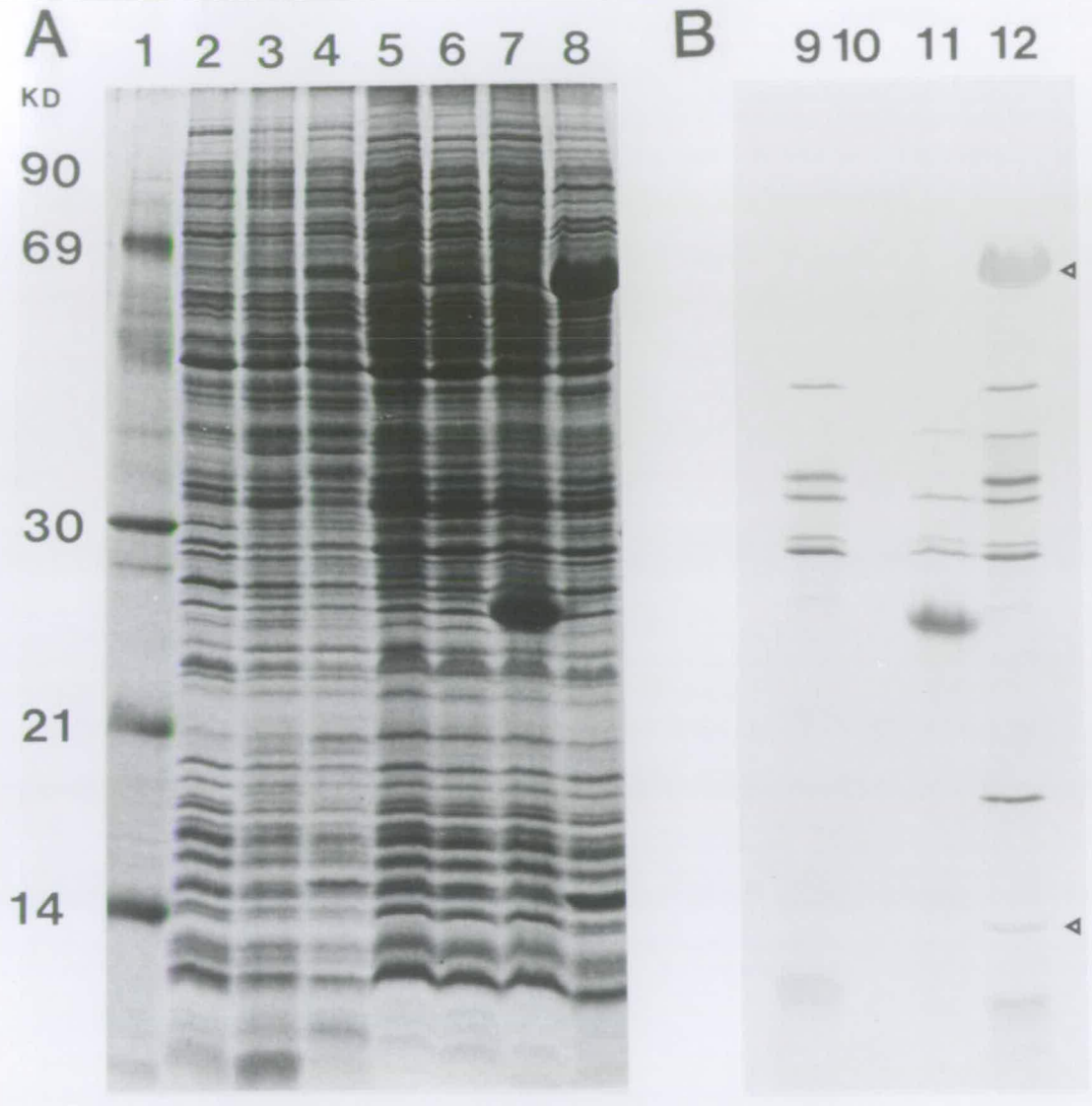
3.4 Plasmid pJM32 appears to be a low copy number plasmid

Two possible explanations could explain this result. The first is that pJM32 does not encode GroES or GroEL, and thus cannot in fact suppress *dnaAts*. This would explain why only one transformant was isolated at 42°C; this being a revertant rather than true suppression by *sdaA*. This would be expected if the *Bam*HI site is present within the *groEL* coding sequence. If this were the case, no suppressing plasmids would result from this cloning experiment. An alternative explanation is that plasmid pJM32 has a low copy number. Thus it might not segregate into minicells, and even in whole cells the extent of GroE over-production would be reduced compared to that seen with pND5. This latter hypothesis was suggested by two observations:-

- When screening the original MM19 transformant and also the later DS410 transformants for the presence of pJM32, very poor plasmid DNA yields were obtained, as might be expected for a low copy number plasmid.

- The transformation frequencies obtained when transforming DS410 with this plasmid were drastically reduced when compared to pJM18 (section 3.3). This might also explain why only 1 out of 100 of the recombinant plasmids (obtained in the original MM19 transformation) was this type (section 3.2).

FIGURE 3.4 Proteins encoded by pND5, pJM18, and pJM32



A Coomassie blue stained whole cell proteins. 14–20% gradient gel.

Track 1: Molecular weight markers: Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsin Inhibitor, Lactalbumin
Track 2: MM19
Track 3: MM19 pJM18
Track 4: MM19 pJM32
Track 5: Minicells pJM18
Track 6: Minicells pJM32
Track 7: Minicells pBR325
Track 8: Minicells pND5

B Autoradiograph of above gel, tracks 5–8. GroE proteins marked with an arrowhead.

Track 9: Minicells pJM18
Track 10: Minicells pJM32
Track 11: Minicells pBR325
Track 12: Minicells pND5

To examine these possiblities, pJM32 was back-transformed into MM19 (*dnaA46ts*) and also into another strain, MM190 (*dnaA606ts*) which was known to be suppressed by *sdaA* (see section 4.3.2). Cells were plated at both 30°C and 42°C. Transformants were isolated at both temperatures, whilst a control experiment using pBR325 only resulted in colonies at 30°C. This provided good evidence that pJM32 was in fact able to suppress *dnaAts*. In addition, the number of transformants obtained was very low, lending further credence to the hypothesis that pJM32 had a low copy number.

TABLE 3.1 Curing MM185 of pJM32: correlation with a return to temperature sensitivity

	30°C		42°C	
	LB	LBamp	LB	LBamp
MM185 pJM32	49	37	37	37
MM185 pJM18	50	50	-	-

The MM185 pJM32 patches unable to grow at 42°C were the same as those unable to grow at 30°C on the ampicillin plate (i.e. all colonies which had lost pJM32 had simultaneously become temperature sensitive).

As a final test of the ability of pJM32 to suppress *dnaAts* mutants, it was decided to cure MM19 of the plasmid and see if temperature sensitivity had returned (to eliminate the possibility that the small number of colonies obtained were revertants rather than suppression). In addition, if the copy number of pJM32 was indeed low, a higher than normal rate of plasmid loss might be expected. Accordingly, fresh overnight cultures of MM19 pJM18 and MM19 pJM32 (grown under antibiotic selection for the plasmid) were diluted 1:5000 into L-broth and grown to stationary phase at 30°C without selection. The cultures were diluted down to give 2-300 colonies per plate and grown up at 30°C on L-agar, again without selection. These were then patched onto ampicillin plates at 30°C and 42°C, to check for plasmid loss and temperature sensitivity. The results are shown in table 3.1. Plasmid pJM32 exhibited a much higher rate of plasmid loss than pJM18; 25% of cells having lost the plasmid by stationary phase compared to 0% for pJM18. In addition, loss of the plasmid is correlated with a return to temperature sensitivity, indicating that pJM32 can without

doubt suppress *dnaAts*. Whilst this high curing rate for pJM32 does not prove that it has a low copy number (since it causes mild cold-sensitivity in MM19 at 30°C -see section 4.4.3- it could be argued that selection is against the plasmid), taken together with the aforementioned results it argues strongly that this is the case.

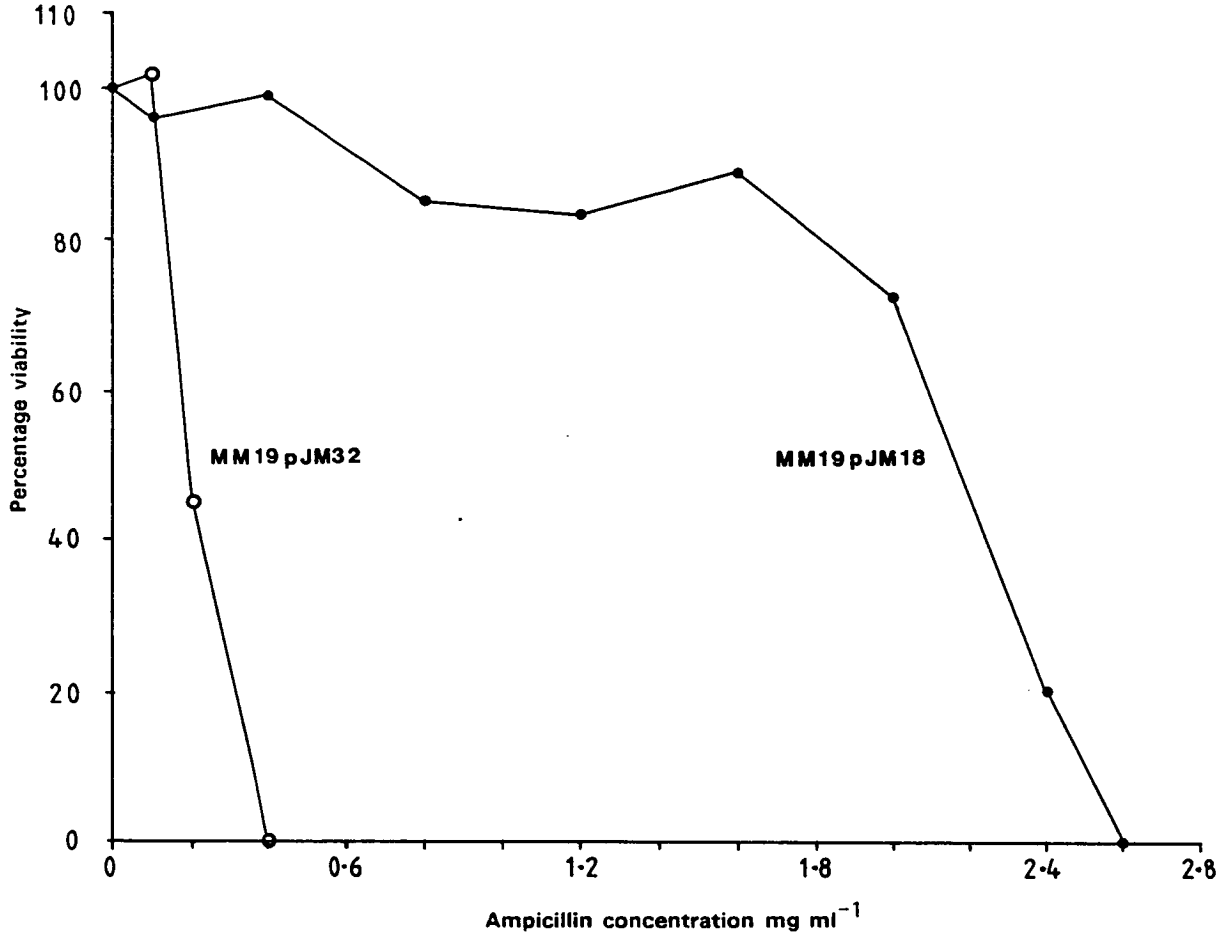
3.5 Measurement of pJM32 copy number

Initial estimates of the copy number of pJM32 were obtained by examining the level of ampicillin resistance conferred by the plasmid. Since a single copy of the β-lactamase gene carried by pJM32 allows resistance to 50μg ml^{-1} of ampicillin, and the level of resistance is fairly linear according to gene dosage (Uhlin and Nordstrom 1977, Uhlin and Nordstrom 1978), this approach should give some indication as to the copy number of pJM32. MM19 cells containing either pJM32 or pJM18 (as a control) were grown to stationary phase in L-broth containing ampicillin at 50μg ml^{-1}. 200μl of a 10^{-6} dilution was plated onto various concentrations of ampicillin and incubated overnight at 30°C. The number of colonies obtained at each ampicillin level were counted and the results are shown graphically in figure 3.5. Resistance levels conferred by pJM18 are high, and are comparable to those obtained with vector (pBR325) alone (see chapter7, and particularly figure 7.12). In contrast, those obtained for pJM32 are very much lower, and assuming that resistance to 50μg ml^{-1} = copy number of 1, this would suggest that pJM32 has a copy number of 3-4 compared to 40-50 for pJM18.

However, caution should be exercised when using such an approach. Since ampicillin resistance is being taken as an indication of copy number, factors which affect the level of ampicillin resistance while not actually affecting the copy number can produce misleading results. For example, pJM32 might contain a strong promoter which could reduce the level of transcription of the β-lactamase gene. Another possibility would be that overproduction of a protein product encoded by the insert in pJM32 might affect resistance, possibly by interfering with the action of β-lactamase in some manner. (Both of these are in fact true to a certain extent; see section 7.2.5 for a discussion of the former effect and section 6.6 for the latter).

Thus, in conjunction with the aforementioned data, although it seemed highly probably that pJM32 did have a low copy number, it was decided to probe directly to the plasmid and measure the copy number by quantitative hybridisation (for a discussion of the technique employed see chapter 9). To guard against an artificially low estimate of copy number due to curing of the plasmid prior to assay (e.g. if 50%

FIGURE 3.5 Ampicillin resistance curves of MM19 pJM18 and MM19 pJM32



Viability at various concentrations of ampicillin compared to L-agar alone. See text for details.

of the culture had lost the plasmid the actual copy number per plasmid-containing cell would be twice that measured), the proportion of plasmid free cells was measured by plating an equal dilution of the culture onto an L-Agar and L-Agar plus ampicillin plate. An approximately equal number of colonies were obtained (532 on L-Agar; 503 on L-Agar plus amp.), indicating that plasmid curing was not a problem. Examination of the results obtained (figure 3.6) confirmed that pJM32 did indeed have a low copy number, only about 15% of that of the vector alone. This was not as low as that suggested by the ampicillin resistance data, but since this was a much more direct measure of plasmid copy number it was assumed to be the more accurate of the two.

FIGURE 3.6 Measurement of pJM32 copy number by quantitative hybridisation

Relative hybridisation levels:- MM185 pBR325 = 740cpm
MM19 pJM32 = 80cpm

Taking into account that pJM32 only contains 73% of vector DNA[a]:-

'Corrected' pJM32 hybridisation figures = $80 \times \frac{100}{73} = 110$ cpm

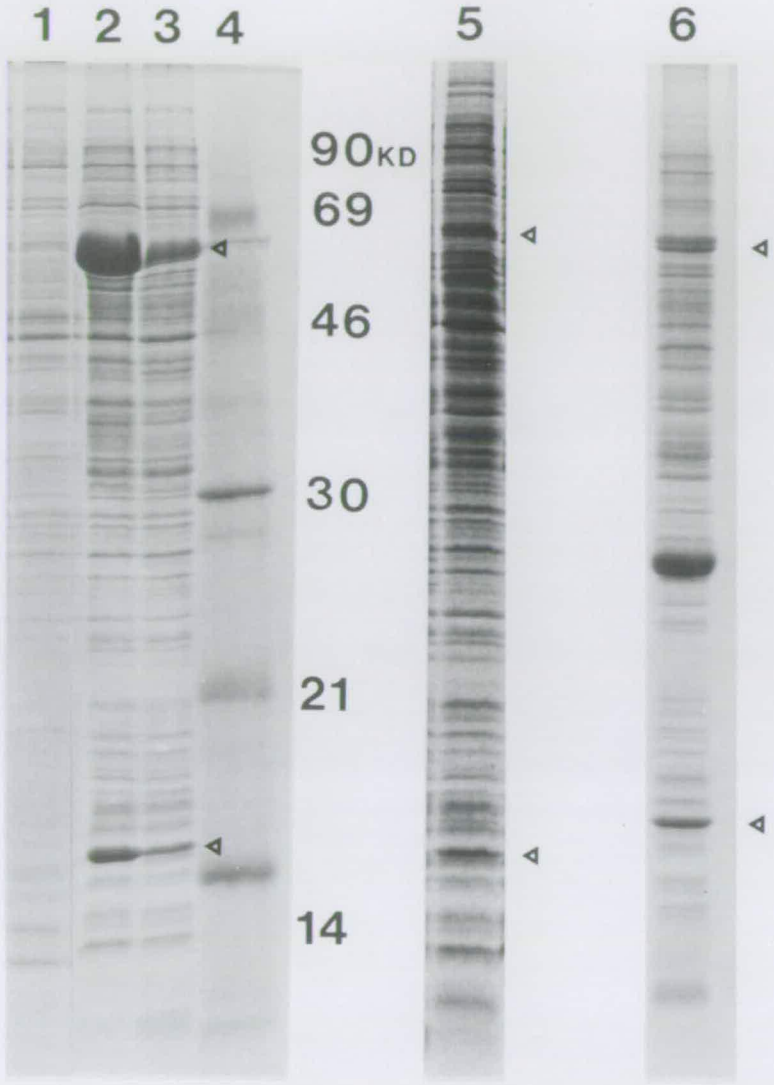
So copy number of pJM32 relative to pBR325 = $\frac{110}{740} \times 100\% =$ **15%**

a. See chapter 8 for full details.

3.6 pJM32 contains a truncated copy of the *groEL* gene

Confirmation that pJM32 is a low copy number plasmid explained why no plasmid encoded proteins had been found in minicells (i.e. the plasmid had failed to segregate into them). However, certain questions remained to be answered. Firstly, is the *groEL* gene product indeed responsible for suppression? Secondly, why is pJM32 ~~is~~ maintained at such a low copy number? Thirdly, and perhaps more importantly, how does it manage to suppress *dnaAts* mutants when very little, if any, extra GroEL protein appears to be present in whole cells? When the copy number of pND5 is reduced to about one third of its normal copy number by a host mutation, it loses the ability to suppress a *dnaA46ts* mutant at 42°C, although it can still suppress at 40°C (this is discussed in section 7.2). Examination of the amount of GroE protein in such a strain reveals a marked reduction compared to strains with a normal copy number, although substantial over-production compared to a strain lacking pND5 (see figure 3.7). Similarly, examining the extent of GroE overproduction in a *dnaAts* strain suppressed by λ *sdaA* at 40°C (the maximum temperature to which this phage was able to suppress a *dnaA46ts* mutant) reveals a reduced amount of GroE protein compared to pND5, but substantially more than present in cells lacking one of these constructs (figure 3.7). Thus, the amount of GroE overproduction seems to determine the temperature to which suppression can occur. Thus the fact that pJM32 was able to suppress *dnaAts* mutants at 42°C with no significant over-production of the GroE proteins was all the more suprising.

FIGURE 3.7 GroE protein levels in strains suppressed by pJM32, pND5 and λ *sidA*



Position of GroES and GroEL proteins marked by an arrowhead. Note that figure is assembled from several gel photographs, therefore magnification factor varies slightly between tracks. 14-20% gradient gel.

Track 1: MM18 dnaA$^+$ 40°C

Track 2: MM185 dnaA46 pND5 40°C

Track 3: JM21 dnaA46 pND5 40°C

Track 4: Molecular weight markers: Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsin Inhibitor, Lactalbumin

Track 5: MM19 dnaA46 pJM32 42°C

Track 6: MM185 dnaA46 pBR325 λsidA 40°C

These questions were partially answered when work by Fayet *et al* (1986) was published, and sequence and restriction map data of the *groE* genes became available (Hemmingsen *et al.* 1988, R. Hendrix, pers. comm.). Work by the former authors, in which they tested subclones of the *groE* region for their ability to suppress *dnaA46ts* mutants showed that suppression was in fact the result of over-expression of both *groES* and *groEL*, and that both gene products were required for suppression to occur. Sequencing data provided an explanation for the low copy phenotype of pJM32. As can be seen in figures 3.2 and 3.8, all of *groES* and about 90% of *groEL* are present on pJM32, and are transcribed from a single promoter reading in the direction *groES-->groEL*. In pJM32 both the chloramphenicol promoter and the promoter for the *groE* genes are reading in tandem into the plasmid origin of replication. Since it is known that transcription into the origin can interfere with plasmid replication (Steuber and Bujard 1982), and from protein gels it can be seen that both genes are very strongly expressed (see figure 3.7), it seems that this could explain the low copy number of pJM32. A feature of the construction of pJM32 (see figure 3.2), was that the orientation of the *Bam*HI-*Eco*RI fragment containing the *groE* genes was reversed with respect to its orientation in pND5. Thus in pND5 the chloramphenicol promoter and the *groE* promoter would actually be reading into each other rather than in tandem into the plasmid origin of replication as is the case in pJM32. This would explain why plasmids with the 8.1kb insert cloned in the opposite orientation in pBR325 proved difficult to maintain in cells (Jenkins 1985).

An examination of the layout of restriction sites in the *groE* genes gave some insights into how pJM32 might be able to suppress *dnaAts* mutations, even though no strong amplification of the GroE proteins is obtained. It can be seen in figure 3.8 that pJM32, although it contains the entire coding sequence of *groES*, has actually lost 10% of the terminal end of the *groEL* gene due to cleavage at the *Bam* HI site. Since the insert in pJM32 is cloned into the *Bam*HI site of pBR325, a *groEL*-tetracycline gene fusion should result. Examining the sequence of both *groEL* and the tetracycline gene reveals that this should result in a fusion protein of 64kd (compared to 60kd for GroEL). In addition, that portion being contributed by the tetracycline gene should be in a different reading frame to the normal tetracycline gene product. Thus we must conclude that this altered GroEL protein is now more efficient at suppressing *dnaAts* mutants, either because the 5' terminus of the *groEL* gene is inhibitory to suppression, or because the change in size of GroEL somehow facilitates reactions with the DnaA protein substrate. Examination of cell proteins in cultures containing pJM32 (figure 3.7) reveals no unique band at 64kd, indicating a low level of expression for this fusion

FIGURE 3.8 Nucleotide sequence of the *groE* operon

Nucleotide sequence of groE operon, and the predicted amino acid sequences of the groES and groEL open reading frames. The BamHI site removing the carboxyl terminus of groEL in pJM32 is marked on the figure. Notice also the met-gly tail of GroEL, absent in the protein encoded by pJM32. Taken from Hemmingsen et al. 1988

protein. Since it is unlikely that the *raison d'etre* of the GroEL protein is to suppress temperature sensitive mutations in the *dnaA* gene, it is conceivable that such a change may render the GroEL protein more efficient at suppressing *dnaAts* mutants (if not in its normal cellular role however). This would possibly tie in with a structural role proposed for the GroE proteins in phage morphogenesis (Kochan and Murialdo 1983, Takano and Kakefunda 1972, Friedman *et al.* 1984), where the size of the protein may be an important determinant of its functional efficiency. Thus although the truncated protein may exhibit a reduced efficiency in its normal cellular interactions, such a change may have rendered it better able to interact with the DnaA protein as its substrate. In the light of this hypothesis, it would be a matter of some interest

then to see what effect changing the size of GroEL had on its ability to suppress *dnaAts* mutations.

3.7 Complementation tests of *groE* mutants by pJM32, pND5 and λ *sidA*

In the light of the ability of this abberant GroEL protein to suppress *dnaAts* mutants, it was considered of some interest to see whether it was capable of complementing chromosomal *groEts* mutants. If the size of GroEL plays an important part in determining its functional activity, an altered form, although more efficient in its interactions with *DnaA*, might be less able to carry out its normal cellular functions. Accordingly, several temperature sensitive mutants in either *groES* or *groEL* were tested for complementation by either λ *sidA*, pJM32, or pND5.

The *groEts* strains (received from Roger Hendrix and Costa Georgopolous; table 2.1) were first tested for their temperature sensitivity on both high salt (L-agar) and low salt (nutrient-agar) media. Since it is likely that *GroE* performs, at least in some instances, a structural role in the cell, the extent of the mutant phenotype might depend on the salt concentration (although GroE possesses an ATPase activity (Hendrix 1979) and therefore might justifyably be regarded as an 'enzyme', it contains regions of homology to the structural protein keratin (see section 3.9.1). (The role of GroE in phage head assembly may be enzymic, with the proteins playing a part in the

TABLE 3.2 Growth and temperature sensitivity of *groEts* strains

	30°C		42°C	
	NA	LA	NA	LA
Colony diameter				
CG25 *groE*$^+$	1.2mm	1.2mm	1.5mm	1.5mm
CG714 *groEL144*	0.5mm	0.8mm	-	-
HX39 *groES619*	0.3mm	0.5mm	-	-
HX369 *groEL44*	0.1mm	0.3mm	-	-
HX24 *groES30*	<<0.1mm	<0.1mm	-	-

NA (Nutrient Agar) is low salt, LA (Luria Agar) is high salt media. Single colonies were streaked using sterile toothpicks and the plates incubated overnight at the appropriate temperature.

reactions underway, or, they may perform a structural role in holding a partially assembled structure together. Alternatively a mixture of both these possibilities may be the true picture). Several examples of such 'salt-reversable' mutant proteins exist, including those likely to have a more structural role in the cell (such as septation proteins; Robinson, Begg and Donachie 1988, Molecular Microbiology in press), as well as proteins more likely to be involved in enzymic activity (including several DNA replication proteins such as DnaB and DnaC; Gross 1972). The results of growth of the *groEts* strains on high and low salt media are shown in table 3.2. *groE* mutant strains grow more poorly than the wild type, even at 30°C, indicating that the mutant protein is not fully functional even at the permissive temperature. The effect is more marked in low salt media, indicating that the mutant phenotype is partially salt reversible. At 42°C no growth is seen on either medium.

The *groEts* strains were then tested for complementation by λ *sidA*, which should introduce a single extra copy of the wild type *groE* genes. This is shown in table 3.3. All strains with the exception of HX24 (*groES30*) are complemented. Lysis of HX24 at 30°C indicates that the strain is λ sensitive, therefore either the temperature sensitive mutation is not in the *groES* gene, or else *groES30* is a dominant allele.

TABLE 3.3 Complementation tests of *groEts* strains using λ *sidA*

	30°C (lysis)	42°C (colonies) *
CG25 *groE*$^+$	+	+
CG714 *groEL144*	+	+
HX39 *groES619*	+	+
HX369 *groEL44*	+	+
HX24 *groES30*	+	–

a. * signifies lysis rather than colonies for strain CG25

b. Tested on L-agar plates c.10^8 phage were spotted onto a lawn of bacteria, with complementation at 42°C being exhibited by dense bacterial growth around the area of the phage spot.

All strains were then transformed with pND5, pJM32 and pBR328 and plated on L-agar plus ampicillin at 50μgml^{-1} to select for transformants. Plasmid pBR328 was

included as a control. Plates were incubated at 30°C and several colonies of each type patched onto identical selective plates and incubated at 30°C and 42°C. Results are shown in table 3.4.

TABLE 3.4 Complementation tests of *groEts* strains using pND5 and pJM32

STRAIN	PLASMID	30°C	42°C
CG25 *groE*$^+$	pBR328	+++	+++
	pND5	+++	+++
	pJM32	+/−	+/−
CG714 *groEL144*	pBR328	++	−
	pND5	+++	+
	pJM32	+/−	+/−
HX39 *groES619*	pBR328	+++	−
	pND5	++	−
	pJM32	+	−
HX369 *groEL44*	pBR328	+++	−
	pND5	+++	+++
	pJM32	+/−	+/−
HX24 *groES30*	pBR328	+++	−
	pND5	+	−
	pJM32	No Transformants Obtained	

Several single colonies of each type were patched onto plates and incubated overnight at the appropriate temperature. Shown is the subjective level of growth compared to a CG25 pBR328 patch grown at 30°C (i.e. a high level of confluent growth = +++).

In keeping with the result obtained for λ *sidA*, HX24 was not complemented by pND5. Indeed, when transformed with this plasmid, growth at 30°C was reduced compared to the control, possibly indicating a deleterious interaction between the mutant GroES30 protein and the wild type GroE proteins at this low temperature

(similar to the cold sensitivity caused by excess GroE in conjunction with certain *dnaAts* alleles (section 4.4)). It was not possible to transform this strain with pJM32. How much this is due to a deleterious interaction between GroES30 and the GroEL-tet protein, and how much is a combination of the general 'sickness' of this strain and the low copy number of pJM32 making transformation difficult cannot be ascertained. In support of this latter hypothesis however, the transformation frequencies obtained for both pND5 and pBR328 into HX24 were very low ($<10^3$ per µg of DNA). Similarly, strain HX39 (*groES619*) was unable to be complemented by either pND5 or pJM32, and growth at 30°C was worse than that of the control. Somewhat suprisingly, this allele had been complemented by λ *sidA*, as though over-expression of *groES-groEL* in conjunction with the mutant *groES619* were preventing complementation at 42°C and causing reduced growth at 30°C. Since this was caused by both pND5 (*groEL*) and pJM32 (*groEL-tet*) it would appear likely this reduced growth/inability to complement is caused by excess GroES protein rather than by the altered GroEL protein. This is further suggested by the data obtained for the two *groELts* alleles tested, in which complementation by GroES-GroEL in high copy number (pND5) *did* occur, and reduced growth compared to the control at 30°C was not observed. Unfortunately, the viability of pJM32 transformants was very poor, either due to the low copy number of the plasmid or possibly due to a deleterious interaction between the two mutant forms of the GroEL protein, and it was not possible to ascertain whether complementation by the GroEL-tet fusion occurred. Bearing in mind the possible role of GroES over-expression in causing reduced growth in a *groESts* mutant strain at 30°C, it would be of interest to see whether over-expression of GroES alone would be enough to cause cold sensitivity in a *dnaAts* strain (section 4.4).

3.8 Effect of salt concentration on the ability of pJM32 to suppress *dnaAts* mutants

Since *groEts* mutants had shown themselves to be at least partially salt reversible (table 3.2), it was decided to investigate whether pJM32 would require a high-salt growth medium for efficient suppression of *dnaAts* mutants to occur. This was in fact the case, and is shown in table 3.5. The effect is not seen in suppression mediated by pND5, indicating that it only occurs with the altered form of GroEL. Thus, although this altered form of GroEL seems to be more efficient at suppressing *dnaAts* mutants, possibly by virtue of a change in the size or conformation of GroEL rendering the DnaAts protein a better substrate for example, suppression by this aberrant GroEL protein requires a higher salt concentration for efficient functioning than does the intact form.

TABLE 3.5 The effect of salt concentration on suppression of *dnaAts* mutations by pNDS and pJM32

	30°C		40°C		42°C	
	NA	LA	NA	LA	NA	LA
Colony diameter						
MM185 pJM32	0.5mm	0.8mm	0.8mm	1.0mm	0.2mm	1.2mm
MM185 pND5	0.2mm	0.2mm	1.0mm	1.0mm	1.2mm	1.2mm

NA is the low salt media, LA is high salt. Cultures streaked to single colonies and incubated overnight at the appropriate temperatures, whereupon colony diameter was measured.

3.9 Discussion and investigation of GroEL protein homologies

The results presented in this chapter describe an attempt to subclone the region responsible for suppression of *dnaAts* mutants (*sdaA/sidA*) from the plasmid pND5. The resulting construct, pJM32, is maintained at a copy number of c.20% of that of the vector alone, presumably due to interference with its replication caused by transcription from the *groE* and chloramphenicol promoters into its replication origin. Although pJM32 contains the complete *groES* gene, about 10% of the C-terminal of *groEL* is missing, being replaced with 10kd of an out-of-frame tetracyline protein. Despite this, efficient suppression of *dnaAts* mutants is accomplished with much lower levels of this protein compared to those required using the intact GroEL.

At least two explanations appear tenable as to why the truncated GroEL protein may function more efficiently than the native form in suppressing *dnaAts*. (1) The deletion may have removed a portion of the protein inhibitory to the specific process of *dnaAts* suppression (although of course this region would not be presumed to be inhibitory to the normal cellular functions of GroEL). (2) An alternative hypothesis is that DnaA is normally a poor substrate for GroEL (assuming the two proteins interact directly). This would explain why such large quantities of GroE are normally required for suppression to occur; not because a large amount of protein is necessary for the actual physiochemical events of suppression *per se*, but to overcome the preference that GroEL has for substrates other than DnaA. Thus all such substrates would tend to 'titrate-out' the available GroEL, requiring that a large amount be available for suppression of *dnaAts* mutations to occur. Removal of some of the GroEL protein sequence may have altered the conformation or size in some manner

such that DnaA is now a much better substrate relative to other cellular proteins. These hypotheses are considered in more detail below.

3.9.1 Deletion of an inhibitory region: homologies between GroEL, keratins, calpains and viral capsid proteins

In view of the first hypothesis; that of the deletion removing a part of the protein inhibitory to suppression, it was decided to examine the deleted region for potentially interesting sequences. Perhaps the most striking feature of this region of the GroEL protein is the string of repeated Gly-Gly-Met residues located at the carboxyl terminus of the protein (see figure 3.8). To investigate a possible significance of this sequence it was decided to search the National Biomedical Research Foundation (NBRF) protein sequence database (version 8; 809 386 residues) for homologies to the sequence GG(GG)X GG(GG)X GG(GG)X GG(GG)X (where X represents any amino acid and the bracketed glycines represent optional residues. This sequence, (rather than the exact sequence present in the GroEL tail) was used for the search to cover a broader range of possible sequence similarities). Further searches were then performed in which the parameters were changed such that X represented any amino acid *other* than glycine in the hope that additional homologies might be revealed. The search was run using the 'Best Local Similarity Algorithim' of Smith and Waterman (1981) as implemented by Lyall *et al.* (1986) on an ICL DAP. Some of the homologies obtained are shown in figure 3.9.

The homologies cover 4 main protein types; keratinous proteins, viral capsid proteins, (calcium dependent) cysteine proteases, and colicins. In the initial search, of the 200 'best-fit' homologies obtained, 180 fell into one or other of these groupings. Also shown are a few protein types which only appeared as single representatives (including *Drosophila melanogaster* and *Xenopus laevis*heat-shock proteins), mainly obtained in the second search.

What might the significance of these glycine rich domains be? Keratins and cytokeratins are subclasses of a large group of proteins known as Intermediate Filaments (IF), which fulfil a 'cytoskeletal' role in the eukaryotic cell (see Steinert *et al* 1985a for review). Immunological and biochemical data have established the existence of a least five subclasses of IF proteins to date, comprising the keratins of epithelial tissue, desmin in muscle, vimentin in cells of mesenchymal origin, a 50kd protein in astroglia, and several neurofilament proteins in neuronal cells. A related protein type, the nuclear lamins, is found in the eukaryotic nucleus, and also exhibits glycine rich

FIGURE 3.9 Proteins containing glycine rich sequences

AMINO-TERMINUS		CARBOXYL-TERMINUS
Protein		**Location in protein[a]**
Keratins		
Search sequence[b]	GGGGXGGGGXGGGGXGGGG	
GroEL[c]	AAGGMGG..MGG..MGGMM	carboxyl
X.laevis 64k type II cytoskeletal	AAGGMGG..gGG..MGGgM	carboxyl
Mouse epidermal keratin	..GGrGGg.sGGg.yGGg.	carboxyl
Pre-high cysteine chorion A	..GGcGcG.cGG..cGG..	amino
Bovine 59k type II keratin	..GGyGG..aGG..yGG..	amino
Bovine elastin A precursor	..GGiGG..vGG..lGv..	amino
Mouse 67k cytoskeletal keratin	.gGGsGGgsyGGg.sGGg.	carboxyl
Human 50k epidermal keratin	.gGGyGGg.lGag.lGGg.	amino
X.laevis ovary cytokeratin	..sGfGGg.yGGg.yGGg.	carboxyl
Polyphemus moth chorion class B	..GGcGG..cdG..wGG..	amino
Viral capsid proteins		
Search sequence[b]	GGGGXGGGGXGGGGXGGGG	
GroEL[c]	AAGGMGG..MGG..MGGMM	carboxyl
M13 and F1 protein A	.gGGsGGg.sGGgseGGg.	centre
Phage FD protein A precursor	.gGGsGGg.sGGgseGGg.	centre
λ capsid protein B	..GyhGGg.sG...fGG..	amino
H1 Parvovirus capsid protein	.gGGssG..gGG..sGGgg	amino
Feline Panleukopenia VP1	.gsGgGG..gGG..sGG..	amino
Murine Minute Virus VP2	..dGpGG..sGGggsGG..	amino
Epstein Barr nuclear antigen	gGGaGG..aGG..aGG..	amino
Feline Parvovirus capsid VP	.gsGgGG..gGG..sGG..	amino
ϕX174 gene H capsid protein	.gaiaGGiasa..laGG..	amino
λ tail protein H	..vGsiGsaiGG.avGGg.	carboxyl
Calpains, colicins, and heat shock proteins		
Search sequence[b]	GGGGXGGGGXGGGGXGGGG	
GroEL[c]	AAGGMGG..MGG..MGGMM	carboxyl
Rabbit; Human; Pig Calpain	ggGGgGGgglGGg.lG...	amino
Streptococcal cysteine proteinase	..G.tGGg.aGGf.nG...	carboxyl
X.laevis hsp70	..GGvpGgmpGs.scGa..	carboxyl
D.melanogaster hsp70	..GGfGG..ysG.....	carboxyl
Chicken embryo hsp70	..GGaG...aGG..sGG..	carboxyl
Colicin E1 protein	.gsGsGG..gGG..kGG..	amino
Cloacin DF13 protein	..GGnsGnhsG..ssGGg.	amino

a. Denotes the location of the glycine rich region within the protein.

b. The X in the sequence signifies any amino acid residue for this position.

c. Exact matches to the GroEL protein sequence are shown in capital letters.

domains (see Franke 1987 for review). All IF subunits exhibit a similar structure. Each is comprised of a central α-helical rod domain of 311-314 amino acids which has a highly conserved secondary structure and which is flanked by end domains of variable size and chemical character (Steinert *et al.* 1985a, 1985b). On the basis of these end domains IF have been divided into several sequence types; Type I (acidic keratins), Type II (neutral-basic keratins), Type III (vimentin, desmin), and the neurofilaments (Hanukoglu and Fuchs 1983, Steinert *et al.* 1984, Geisler *et al.* 1984). The basis for the structural uniformity of diverse Intermediate Filaments is thought to reside in the conserved structure of the central rod domain, while differences in size and properties are thought to be determined by the variable end domains. All IF so far examined are α-type fibrous proteins, with the central conserved region being able to form a coiled-coil α-helical structure. The end domains vary in both size and amino acid content between various IF, although some common features can be recognised. The glycine rich regions of IF are located in these variable end domains, and depending on the individual protein, are located at either the amino, carboxyl or both terminii (see Hanukoglu and Fuchs 1983, Steinert *et al* 1985b). The glycine rich regions of nuclear lamins are located at the carboxyl terminus (McKeon *et al.* 1986, Fisher *et al.* 1986).

A central question concerning this protein type is how the macromolecular cytoskeleton is assembled from the individual precursors, and which domains are important in this assembly. Might the glycine rich region common to GroEL be implicated in this process? It has been noted above that in both IF and GroEL such sequences are located towards the terminal portions of the protein. Biochemical data has indicated that it is the terminal regions of IF proteins that are involved in cross-linkages during cytoskeletal assembly (Steinert *et al.* 1980, Sauk *et al.* 1984, Geisler *et al.* 1982), although the mechanism of such assembly is little understood. It has been suggested (Steinert *et al.* 1985a) that 'registration peptides', comparable to the telopeptides in collagen may be involved in the dimerization process. On the basis of the conserved structure of the central portion of Intermediate Filaments the above authors suggested a specific sequence within this region may fulfil this role. However, might not the glycine rich sequence, strategically situated near the terminal regions be better suited? It has been noted that these glycine rich sequences would be rather hydrophobic, and would therefore tend to make the terminal regions of Intermediate Filaments insoluble. On the basis of this the suggestion that such sequences may interact with a hypothetical 'matrix' protein to form an insoluble structure during assembly has been made (Steinert *et al.* 1984, 1985a). Additionally, in the three dimensional protein structure the terminal domains of IF are predominantly located on the surface of the protein, where they would be available for recognition by a putative

assembly protein (Steinert *et al.* 1983, 1985a). The fact that GroEL acts in the assembly of phage heads and contains a similar sequence would suggest that a glycine rich sequence may indeed act as a point of interaction between the various protein types. In view of this hypothesis, the presence of such a sequence in calpains (calcium-dependent cysteine protease small subunit) is particularly interesting. The function of this small subunit is unknown (Emori *et al.* 1986), but since calpains appear to be specifically associated with cytokeratins (Ishiura *et al.* 1980, Ishizaki *et al.* 1983) it is interesting to speculate that perhaps the proteins may associate via this sequence.

The possibility that a glycine rich domain may function as a 'recognition' sequence or interaction site during macromolecular assembly is further heightened by its occurrence in a variety of viral capsid proteins. Particularly significant is its appearance in the gene B protein of bacteriophage λ, a constituent of the phage capsid and a protein with which GroE is known to interact during phage morphogenesis (Murialdo 1979). Examination of figure 3.9 reveals that glycine rich sequences are also present in a variety of other viral capsid proteins, of both prokaryotic and eukaryotic host viruses, implying that such a sequence may function across a broad range of evolutionary types.

3.9.2 Homologies to the main body of the GroEL protein

How significant is this homology, and does it really represent a recognition/ assembly sequence of some kind, or is there a less revealing explanation behind its presence in any particular protein? What is perhaps particularly striking is that in a search of the entire protein database *only* these protein types showed this sequence; mostly proteins which fulfil some kind of structural role. It may well be significant that proteins which are known to interact or share a common function exhibit this glycine rich sequence. I would like to suggest that this sequence may represent some kind of 'assembly domain', acting as a recognition 'tag' to aid the assembly of macromolecular structures, or possibly being more directly involved in protein cross linkages.

It is of further interest to note that for several of the proteins with which GroEL shares a glycine rich domain, additional homologies between the main body of the proteins exists. This came to light following a computer search of the entire protein database (as described above), but looking for homologies to the whole of the GroEL protein sequence. Such proteins include ϕX174 coat protein, colicin E1 protein,

FIGURE 3.10 Homologies to the main body of the GroEL protein

GroEL protein is shown lowermost; exact matches are denoted by a star above the sequence. Conservative amino acid changes are denoted by a dot.

AMINO-TERMINUS RESIDUE NUMBER		CARBOXYL-TERMINUS RESIDUE NUMBER
	X.laevis 64k cytoskeletal keratin[a]	
	* . .*. .*. * * * *. .*..***** *** ** *	
278	QISNNVSISVISGGSSVYTALG GAAGGMGGGGGMGGGMGGGMG M	321
505	QYAASVAGLMITTECMV TDLPKNDAADLGAAGGMGG MGG MGGM	547
	Carnation Mottle Virus coat protein	
	.** . *. .*. ** *. * ..* * ..* * ** * .*..* ** .	
173	LGKHV ETAAQTAKDLVIPVDGKTRFIRDSASDDAKLVDFGRIVLSTYGFDKADTVVGELFIQYT	236
158	VGKLIAEAMDKVGKEGVITVEDGTG LQDEL DVVEGMQFDRGYLSPYFINKPETGAVELESPF	219
	..* * ..* . . . *. * * ... *.*. * ** . . **. ..* . **	
237	IVLSDPTKTAKISQA SN DKVS DG PTYVVP SVNGNELQLRVVAAGKWCIIVR GTVEG GF	294
220	ILLAD KKISNIREMLPVLEAVAKAGKPLLIIAEDVEGEALATAVVNTIR GIVKVAAVKAPGF	281
	Bacteriophage ϕX174 gene H coat protein[a]	
	** * *. .*.**. .. ** * ** *.. *.. **. . * * *.* *.	
55	AI Q GSNVPNPDEAAPSFVSGAMAKAGK GLLEGTLQAGTSAVSDKLLDLV GL	105
143	AIAQVGTISANSDETVGKLIAEAMDKVGKEGVI TVEDGTG LQDEL DVVEGM	193
	Phage λ minor capsid protein precursor C and capsid assembly protein Nu3	
	... *. *. . . *..* ** * . *.. . . *	
288	DAITVMRD ALDARKSRLSGGRMTKETQSTTVSQTASQADVTDV	330
53	DGVSVAREIELEDKFENM GAQMVKEVASKANDAAGDGTTTATV	104
	.. * *** .*. *. * *.. * ** ** *. . ... *	
331	VP A TEGENA SAA QP DVNAQITAAV AA ENSRIMGILNC	367
105	LAQAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEELKALSV PC	138
	FBJ Murine Osteosarcoma virus envelope polyprotein	
	*** ** * * * * *. . *.*. ***. ..*..* .* * .**	
30	AAGVGTGT TALV A TQQFQQLQAAMHD DLKK VEKSITN LEKSLTSLS	75
84	AAGDGTTTATVLAQAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEE LKALS	135

a. Signifies proteins which also possess a glycine rich sequence (see figure 3.9)

figure 3.10: (continued)
GroEL protein is shown lowermost; exact matches are denoted by a star above the sequence. Conservative amino acid changes are denoted by a dot.

AMINO-TERMINUS RESIDUE NO.		CARBOXYL-TERMINUS RESIDUE NO.
	AKV Murine Leukemia Virus envelope polyprotein	
	*** ** * * * * *. . *.*. *** ..*..* .* * .**	
490	AAGVGTGT TALV A TQQFQQLQAAMHD DLK EVEKSITN LEKSLTSLS	535
84	AAGDGTTTATVLAQAIITEGLKAVAAGMNPMDLKRGIDKAVTAAVEE LKALS	135
	Mouse Polyomavirus coat protein VP1	
	.. . .*. .* .*. * .*. * .* * *	
131	VGSG.SLLDV.HGFNKP.TDTVNT.KVISTPVEGSQ	162
107	VAAGMNPMDLKRGIDKAVTAAVEELKALSVPCSDSK	142
	.* ..... . * *...* * ***.*.	
163	Y HVFAVGGEPLDLQG LVTDARTKYKEEGVVTI	194
143	AIAQVGTISANSDETVGKLIAEAMDKVGKEGVITV	177
	T-cell leukemia virus (HTLV-II) probable protease	
	* ** * .. .*..... . ..* * .*. * . .*.. * * *	
24	LIPLR QQQQPILGVRISVMG QTPQPTQALLDTGADLTVIPQTLVPG	69
426	LADLRGQNEDQNVGIKVALRAMEAPLR QIVLNCGEEPSVVANT VKG	471
	Colicin E1 protein[a]	
	** * . .. *. . * . . .. . * * * * * .	
114	ELAHANNAAMQAEDERLRLAKAEEKARKEAEAAEKAFQE.AEQRRKE	159
308	ELEKATLEDL.GQAKRVVINK.DTTTIIDGVGEEAAIQGRVAQIRQQ	352
	** .. .*. ** . * *.* *. ** * * ..** .**	
160	IEREKAETERQLKLAEAEEKRLAALSEEAKAVEIAQKKLSAAQSEVV	207
353	IEEATSDYDRE KL Q E RVAKLAGGV AV I K VGAA TEV	387
	* * * ..*. ..** * * * .** * . ..* .*	
208	KMDGEIKTLNSRLSSSIHA.RDA.EMKTLAGKRNELAQASAKYKEL	250
388	EMK.E.K..KARVEDALHATRAAVEEGVVAGGGVALIRVASKLADL	429
	X.laevis heat shock protein (hsp) 70[a]	
	..*.* . * * ** .* . .....*. .. . *	
447	ERAMTKDNNLLGKFELSGIP PAPRGVPQIE VTFDIDA	484
121	DKAVTAAVEEL K ALS VPCSDSKAIAQVGTISANSDE	157
	* * *..* **. **. . * * * *. * *	
485	N GILNVSAVEKSSGKQNKITITNDKGRLSKE DI EKM	519
158	TVGKLIAEAMDKV GKEGVITVEDGTG LQDELDVVEGM	193

a. Signifies proteins which also possess a glycine rich sequence (see figure 3.9)

human lamin, mouse polyomavirus, cytokeratin, and perhaps most significantly of all bearing in mind that GroEL is a heat shock protein, the *Xenopus laevis* heat shock protein 70 (hsp70). These homologies are shown in figure 3.10, and would strongly suggest that such proteins share functional similarities. Additionally, the homology seen with the *Xenopus* heat shock protein would suggest that either convergent evolution has taken place between GroEL and hsp70, or else the presence of very strong evolutionary constraints. That such homolgy were to be maintained between organisms as diverse as *Escherichia coli* and *Xenopus laevis* is perhaps little short of remarkable. Homology between hsp70 and the *E.coli* heat shock protein DnaK has been noted before (Bardwell and Craig 1984), although the level of homology between hsp70 and GroEL is considerably lower, and most probably explains why this similarity has not previously been observed.

It is also interesting to note that several of the proteins which share homology with the main body of GroEL (but which lack the glycine rich tail) are also structural proteins (myosin, carnation mottle virus coat protein, phage λ capsid assembly protein Nu3 and capsid precursor protein C). The homology to the λ proteins is particularly interesting since GroE and protein C are known to interact to mediate cleavage of protein Nu3 during assembly of the phage head (Georgopoulos *et al.* 1983). Both proteins Nu3 and C are encoded within the same reading frame, hence the homology to both proteins using only a single sequence.

Several experiments could be performed to test the validity of the hypothesis that a glycine rich tail may be important in protein assembly. Phage types M13, IKe and ϕX174 could be tested to see if they were unable to propagate in a *groE* host (as predicted by the glycine rich regions in some of their coat proteins). Additionally, the truncated GroEL peptide in pJM32 could be tested to see if it were now unable to assemble phages λ and T4. If this were so it would demonstrate that the role of GroEL in the suppression of *dnaAts* mutations is different from its role in macromolecular assembly.

3.9.3 Glycine rich domains as a 'molecular anchor'

The evidence that glycine rich sequences may play a part in the function of structural proteins, either in a recognition or possibly more interactive role has been discussed above. However, why should the removal of such a sequence *increase* the efficiency with which GroE is able to suppress *dnaAts* mutations? At first sight this would appear to be somewhat contradictory. It has been noted above that a glycine rich sequence would tend to be rather hydrophobic in character, and would therefore

tend to make that portion of the protein somewhat insoluble. Such a sequence may result in enforced cross linkages with other proteins, possibly membrane bound, thus reducing the cytoplasmic movement of the protein. Examination of figures 3.9 and 3.10 reveals that bacterial colicins also show these glycine rich tails. The role of these tails in colicins appears to be in membrane interactions. (Masaki and Ohta 1985, Cole *et al.* 1985) Interestingly, experimental evidence has led to the suggest that GroEL is membrane associated (Binowski and Simon 1983). In the role of GroEL as an extrachromosomal suppressor of *dnaAts* the glycine rich tail may decrease the efficiency of this process by anchorage of the GroEL protein; removal of this sequence may allow freer cytoplasmic movement. Regarding the homology between GroEL and the Rubisco subunit binding protein (to be discussed in section 3.9.6), it is interesting to note that although both proteins share a high degree of homology, this glycine rich tail is absent from the latter protein. In *E.coli* GroEL is unable to substitute for the Rubisco binding protein during assembly of Rubisco (the function of this protein being to assemble the Rubisco holoenzyme). This could mean that the Rubisco binding protein has a freer cytoplasmic role than (a possibly membrane bound) GroEL, and it would be interesting to investigate whether removal of the glycine tail might allow GroEL to assemble Rubisco in *E.coli*.

3.9.4 Over-expression of GroEL is able to suppress temperature sensitive mutations in the *ams* gene

The ability of a truncated GroEL protein to suppress *dnaAts* mutations is not without precedent, and some insights into this phenomenon may be suggested by the results of Chanda *et al.* (1985). Their work involved an attempt to clone the *ams* (alteration of messenger RNA stability) gene of *E.coli*, which had been mapped to 23 minutes on the genetic map (Ono and Kuwano 1980). A temperature sensitive mutant unable to grow at 42°C existed, which was characterised by a mRNA half life of 10-12 minutes, compared to 0.5-2.0 minutes for the wild type (at 42°C), and the authors hypothesised that perhaps the *ams* gene encoded an RNase involved in the degradation of messenger transcripts.

An attempt to isolate the *ams* gene from an *Eco*RI library of chromosomal DNA cloned into the *Eco*RI site of pBR322 resulted in the isolation of an 8.1kb fragment seemingly able to complement the *ts* phenotype and to allow growth at 42°C. mRNA stabilty in such 'complemented' mutants was reduced to nearly wild type levels; namely a half life of 3-4 minutes. Subclones of this 8.1kb insert were tested for complementation in an attempt to delimit the region responsible for *ams* complementation to that encoding a specific gene product. In doing so they they

identified a 600bp 'minimal complementing unit' which they duly sequenced. Sequencing data showed an open reading frame capable of encoding a 17kd protein, which they visualised on protein gels. As a further check the reading frame of the putative Ams protein was, altered, and an 11kd form no longer able to complement was observed.

What they had in fact cloned was an internal fragment of the *groEL* gene, i.e. extrachromosomal suppression of the *ams* gene. That *groEL* itself is not the *ams* gene is evident from their widely differing map locations; 23 minutes for *ams* and 94 minutes for *groEL*. Assuming these results are sound, this demonstrates three important points about GroE suppression. Firstly, over-expression of GroE is able to suppress at least one other *ts* mutant besides *dnaA*. Secondly, in contrast to *dnaAts*, supression of *ams* does not require over-expression of *groES*, possibly indicative of a different mechanism. Thirdly, it would appear that suppression of *ams* can be accomplished by an even smaller portion of the *groEL* gene than is present in pJM32. Perhaps the most interesting aspect of all this is that the 17kd GroEL protein fragment was considerably more efficient than the full sized GroEL at suppressing *ams*; reducing the mRNA half life to 2 minutes as compared to 4 minutes for the full sized protein. This would tie in with the results obtained with pJM32 in which the truncated GroEL peptide appears to function more efficiently as a suppressor of *dnaAts* than does the full sized protein.

3.9.5 Deletion of an inhibitory region or the alteration of substrate specificities?

Thus for the suppression of both *ams* and *dnaAts* mutations by over-expression of the *groE* genes the process can be seen to be more efficient using a truncated version of the GroEL protein. Is this due to the deletion of an inhibitory region of GroEL, or a result of changes in the substrate specificities of the altered protein? The removal of the glycine rich sequence in the GroEL-tet fusion protein, together with the presence of this sequence in a variety of cross-linked or membrane bound proteins would probably tend to suggest the former hypothesis in this instance. As a more definitive test of this, a plasmid could be constructed in which the *groEL* gene has been truncated at the *Bam* H1 site as per pJM32, but in which translational stop codons have been inserted to prevent incorporation of the *tet* gene tail. If such a construct was still able to efficiently suppress *dnaAts* mutations, the conclusion would be that removal of an inhibitory region was the most likely cause for the efficient suppression mediated by pJM32. If not, the likely explanation would be that the presence of the *tet* gene tail were necessary, presumably in allowing the protein

to adopt a favourable conformation.

The effect seen with GroEL suppression of *ams* is somewhat different, since the smallest protein fragment (17kd compared to a full size of 58kd) resulted in the most efficient suppression. It seems unlikely that this can be explained by simple deletion of an inhibitory region, since larger fragments of GroEL, although lacking the glycine tail and more efficient at suppression than the full size protein, were less efficient than this smaller fragment. In the case of *ams* suppression at least, this would suggest that the conformation of the smallest GroEL fragment is a major determinant of the efficiency of this process. It would perhaps be of value to investigate whether smaller fragments of GroEL were able to suppress *dnaAts* mutations also. In the case of *ams* suppression at least, this provides strong evidence that GroEL suppresses by some specific structural interaction with the gene product of interest. Only by such a mechanism can it easily be explained how altering the size (conformation) of the GroEL protein can result in more efficient suppression; namely by rendering the suppressable protein a better substrate. If suppression were mediated by a more general mechanism, for example by increasing transcription/ translation as a result of interactions with RNA polymerase or ribosomes -interactions which normally occur in the cell (Neidhardt *et al.* 1981, Ishihama *et al.* 1976, Wada *et al.* 1987)- it is hard to conceive of how alterations to the size of the GroEL protein could render this process more efficient. Indeed, it would be expected that a truncated GroEL protein would be less efficient at fulfiling its normal cellular functions (although under this hypothesis more efficient in its role as a suppressor).

3.9.6 The role of GroE as a Molecular Chaparone: homology between GroEL and the Rubisco subunit binding protein

Such a structurally interactive role has recently been postulated for the GroE proteins on the basis of amino acid homology to another protein thought to be involved in protein folding and assembly, the Rubisco large subunit-binding protein of *Ricinus communis* (castor bean; Ellis, 1987, 1988, Hemmingsen *et al.* 1988). In addition, homology has been demonstrated with a major 65kd antigen of *Mycobacteria leprae* and *M.tuberculosis*, antibodies raised against this antigen cross reacting with GroEL (Young *et al.* 1987, Young *et al.* 1988). The role of this 65kd protein in *Mycobacteria* has not been ascertained, although it appears to be a major immunogen in a number of mycobacterial infections (Young *et al.* 1987). The observed homologies extend throughout the GroEL, Mycobacterial, and Rubisco (binding) proteins, unlike that noted earlier in which homology to specific domains only was seen. These high levels of homology between *Mycobacteria, E.coli* and plant proteins

would surely argue that severe evolutionary constraints exist on this particular protein type. In addition,the occurrence of GroE-like proteins has been reported in the mitochondria of yeast, maize and man (McMullin and Hallberg 1988, quoted in Hemmingsen *et al.* 1988).

The role of this class of protein has been proposed by Ellis to be that of a 'molecular chaperone', ensuring the correct folding of certain polypeptide chains, and their subsequent assembly into oligomeric structures. He suggests that in some instances transient interactions between hydrophobic or charged surfaces of proteins can lead to the assembly of incorrect structures. The role of the 'molecular chaperone' would be to prevent the formation of such structures. It is also suggested that these proteins can disassemble such structures and unwind incorrectly folded peptides that might be formed during stresses, such as heat shock for example. Both functions would provide a plausible role for GroE in the suppression of *dnaAts* mutations. Initiation of DNA replication at *oriC* requires the assembly of a range of proteins into an 'initiation complex' (see Kornberg 1988). GroE might be involved in the formation of this complex, thus suggesting an obvious mechanism of suppression by GroE; increased amounts possibly compensating for a reduced activity/stability of one of the constituent proteins. However, if this is the case GroE would be expected to suppress other initiation mutants such as *dnaB* or *dnaC* for example. To date, out of those initiation mutants tested, only *dnaAts* mutations have been found to be suppressed by GroE (Jenkins *et al.* 1986, Fayet *et al.* 1986), although since only a small number of mutant alleles of each type have been tested, a systematic examination involving a greater number of alleles may show suppression to be possible in some cases. This model assumes that GroE performs a role in the initiation of DNA replication under normal conditions, a hypothesis supported by the finding that *groE* mutants fail to replicate their DNA properly at the restrictive temperature (Tilly *et al.* 1981, Wada and Itikawa 1984). However, it is difficult to explain why the mutant form of the GroEL protein (pJM32) should function more efficiently in this process than the full-sized protein if it is a normal intracellular interaction.

In the light of a possible role for GroE in disassembling incorrectly folded proteins, it is possible that suppression occurs by the unfolding of an incorrectly formed protein at high temperature. This would assume that in the absence of excess GroE a high proportion of the DnaAts protein would be incorrectly folded. This might explain why over-production of mutant DnaA protein allows growth at high temperature (quoted in Atlung, 1984); the small proportion of undenatured protein

being raised to physiologically active levels. Under this model, since DnaA is a poor substrate for the native GroEL protein, large quanities would need to be present for this unfolding to occur, since competetion from more suitable substrates would effectively titrate out all the GroEL available. The altered GroEL present in pJM32 now means that DnaAts is a much better substrate, and as such, much lower levels are required for suppression to occur. In addition, competition from normal cellular proteins which interact with GroEL is now eliminated since they are of the wrong size to interact with this altered GroEL.

The evidence that such 'molecular chaperones' exist is good. The role of the GroE proteins in phage morphogenisis is to ensure correct assembly of capsid protein by transient binding during assembly whilst not actually being part of the mature structure. GroEL has a known ATP-ase activity (Ishihama *et al* 1976, Hendrix 1979), and purified multi-subunit complexes between GroES and GroEL are reported to dissociate upon addition of ATP (Douglas Young, pers. comm.), although in a somewhat contradictory fashion it has also been reported that the presence of Mg-ATP is required for such complexes to form (Chandrasekhar *et al.* 1986). Perhaps this is an example of the 'complex dissociation role' postulated for these molecular chaperones (Hemmingsen *et al.* 1988), with this presumably being an ATP dependent process. Rubisco binding protein seems to play a similar role in *Ricinus comminus*, being involved in the assembly of the rubisco enzyme complex (Barraclough and Ellis, 1980; Musgrove *et al.*, 1987). It is interesting to note however that GroEL is unable to substitute for the Rubisco binding protein in the assembly of the Rubisco complex in *E.coli* (quoted in Hemmingsen *et al.* 1988), suggesting that differences between the bacterial and plant 'chaperones' are sufficiently pronounced for this to be so.

Is there such a protein class as 'molecular chaperones'? Such a question cannot be answered at present, but it is perhaps significant that of the three classes of chaperone suggested by Hemmingsen *et al.*, namely, (1) nucleoplasmin, (2) hsp70-immunoglobulin heavy chain binding protein class, and (3) bacterial-mitochondrial-chloroplast class, both of the latter classes show some degree of homology to GroEL at the amino acid level. Hemmingsen *et al.* used the observed homology to GroEL to delimit the latter class; on the basis of the homologies reported here it may be more accurate to consider (2) and (3) as representative of a single class. However, definitive proof that such protein types exist, and the importance of such processes in GroE suppression of *dnaAts* mutations requires further work. For the latter question at least, this may involve the use of purified mutant DnaA protein and an examination of whether the addition of GroE could allow formation of an active initiation complex.

Addressing a possible role in unwinding an abberrantly folded DnaA protein, it should be possible to discover whether addition of GroE restores functional activity to an otherwise inactive form, subsequent removal of the GroE demonstrating this to be a transient interaction. The question of GroE acting to 'repair' inactivated DnaA is addressed more fully in chapter 5.

Having discussed the evidence that GroE suppression may be mediated by a specific interaction with the substrate protein, I will now describe experiments conducted to examine other possible mechanisms by which over-expression of the *groE* genes is able to suppress *dnaAts* mutations.

CHAPTER 4

Investigation into possible methods of suppression of *dnaAts* mutants by over-expression of the *groE* genes

4.1 Introduction

The experiments described in this chapter are essentially concerned with a genetic investigation into the mechanism by which suppression may be occurring. They include an investigation into the allele specificity of suppression and an examination of the effect of pND5 upon transcription of the *dnaA* gene. Also included is a detailed investigation into the cold sensitivity caused by the presence of pND5/λ *sidA* in combination with certain *dnaAts* alleles. This is included here (rather than in chapter 6), because of the specific nature of the effect. The pattern of allele specificity is similar to that present in *dnaAts*/*dnaA*$^+$ merodiploids, suggesting that it may be an integral part of the mechanism by which suppression is occurring. The data in chapter 6 concern phenotypic side-effects of pND5/λ *sidA* which are of a more general nature, and are not included here for the sake of clarity.

4.2 Suppression is not bypass in nature

Bypass suppression occurs when the product of the suppressing gene can substitute for the defective protein, or bring into action an alternative pathway which does not require it. That is, it alleviates the need for the protein. In these cases amber mutations are suppressible. In *E.coli* at least two mechanisms of bypass suppression for *dnaA* exist. (1) Integrative suppression occurs when replication initiates from an integrated plasmid origin which does not require the DnaA protein (Molin and Nordstrom 1980). (2) In stable DNA replication (*sdrA*) mutants of *E.coli*, initiation occurs from a variety of secondary origins located around the chromosome (Ogawa *et al.* 1984). This can occur in an *rnh* mutant, (encoding RNAaseH), which is normally involved in the degradation of the RNA moiety of RNA:DNA hybrids. Indeed, it is possible that the normal role for this protein *in vivo* is to prevent initiation from such secondary origins occurring.

Accordingly, strains NS387 (*dnaA311am*) and NS388 (*dnaA366am*) were transformed with the *groES-groEL* containing plasmid pND5 (section 3.2) and tested for growth at high temperature. Both strains are suppressed at low temperature by the temperature sensitive conditional suppressor *supF81*. Plasmid pBR328 was included as a negative control, and plasmid pJM87-01 as a positive control. This latter plasmid (described in section 5.1.4) contains the intact *dnaA* gene and its promoter. The results are shown in table 4.1. Plasmid pND5 is unable to suppress *dnaAam* mutations, indicating that *groE* suppression is not bypass in nature and it requires the presence of the mutant DnaA protein.

TABLE 4.1 Testing pNDS for suppression of *dnaAam* mutations

STRAIN	PLASMID	30°C	42°C
NS387 *dnaA311am*	pBR328	+	-
	pND5	+	-
	pJM87-01	+	+
NS388 *dnaA366am*	pBR328	+	-
	pND5	+	-
	pJM87-01	+	+

Ability of each strain to grow at the respective temperature on LBamp50 plates.

4.3 Allele specificity of *dnaAts* suppression by *groE*

Important indications into the nature of suppression can often be obtained by an examination of the allele specificity exhibited. For example, suppression of a clustered group of alleles can suggest the presence of a functional domain within a protein, possibly that section which interacts with the suppressing protein. In addition, a pattern of allele specificity identical to that exhibited by a different suppressor of the same gene can indicate a similar mechanism. Finally, if the suppressible alleles are all the same kind of mutation (e.g. the same missense mutation), then this argues strongly for some kind of informational suppression, such as the classic tRNA informational suppressors. For this reason, it was decided to examine the pattern of allele specificity exhibited by *groE* suppression of *dnaAts*.

4.3.1 Construction of isogenic *dnaAts* strains

In order to give comparable results it was decided to transfer each *dnaAts* allele into the same background and test for suppression. This was done by cotransduction with *asnA*$^+$ into MM18. Before transfer, each donor strain was tested for temperature sensitivity and complementation by λ*dnaA* (λ425 of Schaus *et al.*, 1981a). In addition, each strain was tested for suppression by *groE* using λ*sidA* (this phage is described in section 3.2). This was intended to give preliminary data on the

pattern of allele specificity and hopefully eliminate differences in suppression as a result of a different genetic background when transferred into MM18. These results are shown in table 4.2. None of the strains grow at 40°C, confirming their temperature sensitive phenotype. All strains are lysed by λ *dnaA* at 30°C and complemented at 40°C. In addition, all alleles with the exception of *dnaA203ts, dnaA204ts* and *dnaA508ts* are suppressed by λ *sidA*. The *dnaAts* alleles were then transferred into MM18 by transducing to *asn*$^+$ using lysates made on these strains, followed by screening for temperature sensitivity. The resultant isogenic *dnaAts* strains were called MM181-190.

TABLE 4.2 Complementation/Suppression of *dnaAts* alleles before transfer into MM18

STRAIN	MUTATION	COMPLEMENTATION BY λ *dnaA* [a]	SUPPRESSION BY λ *sidA* [a]
WM1029	*dnaA167*	+	+
LC905	*dnaA5*	+	+
WM1152	*dnaA204*	+	-
WM1032	*dnaA508*	+	-
WM1026	*dnaA46*	+	+
CM748	*dnaA203*	+	-
CM2733	*dnaA602*	+	+
CM2735	*dnaA601*	+	+
CM2738	*dnaA604*	+	+
CM2740	*dnaA606*	+	+

a. Growth of colonies within phage spots at 40°C. All strains were confluently lysed at 30°C. None of the strains were able to grow at 40°C in the absence of complementation or suppression.

4.3.2 Testing isogenic *dnaAts* strains for suppression by *groE*

Strains MM181-190 were then tested for suppression by λ *sidA* and pND5 at both 40°C and 42°C. λ *dnaA* was included as a control to ensure that the *ts* phenotype was due to transfer of the mutant *dnaAts* allele. These results are shown in table 4.3. All strains are complemented by λ *dnaA*, indicating correct transfer of the *dnaAts* allele. The pattern of allele specificity seen in table 4.2 was repeated here, indicating that it was not a strain specific effect. Some alleles are only able to grow up to 40°C

TABLE 4.3 Complementation/Suppression of *dnaAts* alleles in isogenic MM181-190 series

STRAIN	MUTATION	COMPLEMENTATION BY λ *dnaA*[a] AT	SUPPRESSION BY λ *sidA*[a] AT		SUPPRESSION BY pND5 AT	
		42°C	40°C	42°C	40°C	42°C
MM181	*dnaA167*	+	+	+	+	+
MM182	*dnaA5*	+	+	–	+	+
MM183	*dnaA204*	+	–	–	–	–
MM184	*dnaA508*	+	–	–	–	–
MM185	*dnaA46*	+	+	+	+	+
MM186	*dnaA203*	+	–	–	–	–
MM187	*dnaA602*	+	+	–	+	–
MM188	*dnaA601*	+	+	–	+	–
MM189	*dnaA604*	+	+	–	+	–
MM190	*dnaA606*	+	+	+	+	+

a. Growth of colonies within phage spots at 40°C or 42°C. All strains were confluently lysed at 30°C. None of the strains were able to grow at either 40°C or 42°C in the absence of complementation or suppression.

irrespective of whether suppression is by pND5 or λ *sidA*. The pattern of alleles which can be suppressed up to 40°C (figure 4.1) is interesting, since all four alleles are located adjacent to each other in the *dnaA* gene. This may represent a functional domain or point of interaction between GroE and DnaA perhaps; alternatively, mutations in this portion of the gene may have a more severe effect upon the ability of the protein to function. In strain MM182 (*dnaA5*), growth with λ *sidA* was only possible up to 40°C; when transformed with pND5 however growth at 42°C was possible. Since suppression by λ *sidA* is a *recA* dependent process (Jenkins, 1985), and involves multiple lysogenisation of the phage (Fayet *et al.*, 1986), this probably reflects an increased amount of GroE present in the plasmid containing strain (compared to the lysogen), allowing growth at this higher temperature (compare GroE protein levels in MM185 suppressed with pND5 or λ *sidA* in figure 3.7). As a test that suppression by *groE* is in itself not a *recA* dependent process, strain MM22 (MM18 *recA* *dnaA46ts*) was transformed with pND5 and pBR325 (as a control) and

tested for growth at 42°C. This is shown in table 4.4. MM22 pND5 is able to grow at 42°C in contrast to MM22 pBR325, and confirms that suppression can indeed occur in a *recA* strain.

TABLE 4.4 Suppression by pND5 in a *recA* background

	30°C	32°C	40°C	42°C
MM22 pNDS	+	+	+	+/−
MM22 pBR325	+	+/−	−	−

Single colonies of each were streaked on LBamp50 plates and incubated overnight at the appropriate temperature.

4.3.3 The pattern of allele specificity exhibited by *groE* suppression is identical to that of certain *rpoB* mutants

As has been discussed in chapter 1, a considerable number of extragenic suppressors of *dnaAts* exist. These can take the form of cloned suppressors which can presumably act as such due to their new molecular environment (of which *groE* is an example), or alternatively, suppression can be due to secondary mutations in another gene. Of this class, probably the most intensively studied is that involving secondary mutations in the *rpoB* gene, which codes for the β subunit of RNA polymerase.

All *dnaAts* alleles so far studied can be suppressed by secondary mutations in the *rpoB* gene; however, each *rpoB* mutant displays allele specificity as to which of the *dnaAts* mutations it will suppress (Atlung 1984). In addition, one of the *dnaAam* mutations is suppressed by *rpoB* (*dnaAam311* -this allele was not one of those tested for suppression by *groE*- Schaus *et al.* 1981a), suggesting that suppression may be informational or bypass in nature. However, since other *dnaAam* mutants are not suppressed, at least a partially functional DnaA protein is probably required for suppression to occur. This is further suggested by the position of the suppressed amber mutation within the gene; mapping to the distal end, and as such leaving most of the protein intact (Schaus *et al.* 1981b).

A comparison of the map position of various temperature sensitive alleles of *dnaA* with the pattern of suppression by *rpoB* and *groE* reveals some highly

interesting correlations (figure 4.1). Firstly, the pattern of allele specificity exhibited by *groE* suppression is exactly matched by that of four of the *rpoB* alleles (*rpoB902, rpoB909, rpob911* and *rpoB918*). Secondly, all of these suppressed alleles are located in the central portion of the *dnaA* gene; both *groE* and the aforementioned *rpoB* alleles are unable to suppress those mutations mapping to the ends of the *dnaA* gene. These peripheral mutations can be suppressed by *rpoB*, but a different allele is required for each end. In addition, the *rpoB* alleles which suppress these peripheral *dnaA* mutations are themselves unable to suppress those located in the central portion of the gene. This would seem to suggest that the mechanism of suppression between alleles located in the central and peripheral portions of the *dnaA* gene is somewhat different. In the case of *rpoB* at least, this would appear to suggest some kind of interactive suppression, especially since both DnaA and RNA polymerase are known to act at an early stage in the initiation process (Fuller and Kornberg 1983, Zyskind *et al.* 1977). In addition, this would suggest two, and possibly three different functional domains within the DnaA protein, each one subject to a different kind of suppression. However, it should be remembered that a two dimensional linear map of allele location within a gene need not necessarily bear much resemblance to the location within the three-dimensional protein structure. Regardless of this, the correlation still appears striking.

FIGURE 4.1 Pattern of allele specificity of suppression by over-expression of *groE* and by secondary mutations in *rpoB*

	dnaAts allele no. (location with respect to promoter of *dnaA* *dnaA* ← P)									
	203	*204*	*602*	*601*	*604*	*606*	*5*	*46*	*167*	*508*
rpoB[a]	–	–	+	+	+	+	+	+	+	–
groE[b]	–	–	+	+	+	++	++	++	++	–

a. Signifies suppression of dnaAts mutations by secondary mutations in rpoB gene, allele nos. rpoB902, rpoB909, rpoB911, rpoB918. Data and location of dnaAts alleles taken from atlung 1984, Hansen et al 1984.

b. Signifies suppression of dnaAts muations by over-expression of the groE genes. + = Suppression up 40°C; ++ = suppression up to 42°C.

The identical pattern of allele specificity between suppression by over-expression of *groE* and by secondary mutations in the *rpoB* gene suggests a similar mechanism. Interactive suppression has already been mentioned; DnaA and RNA polymerase may interact as part of an initiation complex. The identical pattern of allele specificity exhibited by *groE* suppression might indicate a role in the assembly/maintainence of such a structure. (The fact that *groE* mutants fail to replicate their DNA properly could be due to the non-assembly of such a complex for example). This presumes that suppression in this instance is due to a direct interaction between DnaA and GroE. Another possibility is that suppression by *groE* is due to an interaction between RNA polymerase and GroE affecting transcription of the *dnaA* gene. It has been reported (quoted in Atlung 1984) that over-expression of mutant DnaAts protein leads to a suppression of temperature sensitivity. Therefore it is possible that extra GroE is causing the *rpoB* gene product to act as a mutant form, and that this mutant form is behaving like alleles *rpoB902, rpoB909, rpoB911* and *rpoB918*, which, under this model, are presumed to suppress by increasing the transcription of the *dnaA* gene. GroE has been reported to co-purify with RNA polymerase (Ishihama *et al.* 1976a, 1976b), so some kind of interaction, either during initiation or in a more general transcriptional sense does seem plausible.

4.3.4 Over-expression of *groE* does not cause rifampicin resistance

As an initial indication of the nature of the interaction between GroE and RNA polymerase, it was decided to examine whether over-expresssion of *groE* conferred rifampicin resistance upon a normally sensitive host. The suppressing *rpoB* alleles described above had originally been isolated by selecting for rifampicin resistance followed by screening the resultant mutant alleles for their ability to suppress *dnaAts*. Thus all suppressing *rpoB* alleles were also rifampicin resistant. If suppression by *groE* is due to the extra protein interacting with RNA polymerase and altering the transcription of certain genes, this might lead to rifampicin resistance. Accordingly, a strain over-expressing *groE* (MM18 pND5), together with positive (Toe12 pBR325) and negative (MM18 pBR325) controls were tested for rifampicin resistance. As can be seen in table 4.5, pND5 does not cause resistance to rifampicin in a previously sensitive strain. Excess GroE is evidently not altering RNA polymerase so as to cause the rifampicin resistance seen with the *rpoB* suppressors of *dnaAts* then. However, this is not to say that GroE does not suppress via an interaction with RNA polymerase, nor does it preclude the possibility that suppression may be due to increased expression of the mutant *dnaAts* gene. This is examined further in section 4.5 using *dnaA-lacZ* translational fusions.

TABLE 4.5 pND5 and rifampicin resistance

STRAIN	PLASMID	LA	LArif
TOE12	pBR325	+	+
MM18	pBR325	+	-
MM18	pND5	+	-

Single colonies were streaked onto LA (Luria Agar) plates with or without rifampicin (rif), and incubated overnight at 37°C.

4.4 Cold sensitivity caused by *groE* over-expression

A notable feature of *groE* over-expression is the cold sensitive phenotype exhibited in conjunction with many *dnaA* alleles. This is characterised by a much reduced growth rate at 30°C compared to controls, and occurs with both pND5 and λ *sidA*. In contrast to the former, growth at 30°C with λ *sidA* lysogens results in a very heterogeneous mixture of colony sizes, presumably reflecting differences in the level of multiple lysogenisation by the phage. As such, quantitative studies on this phenomena were conducted using pND5 containing strains.

At this point a distinction between cold sensitivity and a general reduction in growth due to over-expression of *groE* should be made. In many strains pND5 causes a general reduction in growth rate and the degree appears to be strain specific (this is covered more thoroughly in chapter 6). However, this occurs at all temperatures and the reduction is generally small. Since strains containing pND5 produce considerable quantities of the GroE proteins (see figure 3.4, this may reflect a strain imposed upon the cells' metabolism in producing such quantities rather than any specific deleterious effect. In contrast, some *dnaAts* strains containing pND5 experience a very severe reduction in growth rate at 30°C compared to *dnaA*$^+$ controls, this effect disappearing as the temperature is raised (when even more GroE should be produced in the cell). Cold sensitivity is phenotypically quite different in effect then, and since the *dnaAts* strains (MM181-190) are isogenic with the *dnaA*$^+$ control (MM18), any reductions caused by pND5 must be due to a specific effect on that allele.

TABLE 4.6 Growth of *dnaAts* strains in conjunction with pND5: cold sensitivity

STRAIN		ALLELE	30°C	37°C	40°C	42°C
COLONY DIAMETER (MM)						
MM18	pND5	*dnaA*$^+$	1.0	1.5	1.5	1.5
	pBR325		1.0	1.5	1.5	1.5
MM181	pND5	*167*	1.0	1.3	1.5	1.5
	pBR325		1.3	1.5	<0.1	-
MM182	pND5	*5*	<0.1	0.1-0.3	1.3	1.0
	pBR325		1.0	-	-	-
MM183	pND5	*204*	0.8	1.3	-	-
	pBR325		1.0	1.5	-	-
MM184	pND5	*508*	0.8	1.0	-	-
	pBR325		1.0	1.5	-	-
MM185	pND5	*46*	0.1-0.3	1.0	1.5	1.5
	pBR325		1.0	-	-	-
MM186	pND5	*203*	0.1	1.0	-	-
	pBR325		1.0	1.5	-	-
MM187	pND5	*602*	-	0.3	1.3	-
	pBR325		1.0	-	-	-
MM188	pND5	*601*	-	0.3	1.3	-
	pBR325		1.0	-	-	-
MM189	pND5	*604*	-	0.3	1.3	-
	pBR325		1.0	-	-	-
MM190	pND5	*606*	-	0.1	1.3	1.0
	pBR325		1.0	0.5	-	-

Cultures streaked to single colonies on LA tetracycline plates and incubated overnight (c.18h) at the respective temperature, whereupon colony diameter was measured. A minus sign (-) indicates no growth.

4.4.1 Cold sensitivity is allele specific

Cold sensitivity is characterised by a much reduced growth rate at 30°C for certain *dnaAts* alleles in conjunction with pND5. The easiest way of visualising this is

shown in table 4.7. Examining MM185 pBR325 (*dnaA46ts*), it can be seen that growth proceeds well at 30°C, as would be expected. At both 37°C and 42°C a gradual cessation of growth occurs, the effect being more pronounced at the higher temperature. Similar results are obtained with MM182 pBR325 (*dnaA5ts*), except that complete cessation of growth at 37°C with this allele occurs. Examination of the cultures grown at 42°C under the light microscope revealed long uninucleate filaments and a considerable number of cell 'ghosts' and debris.

TABLE 4.7 Effect of pND5 on growth rates of strains MM182 and MM185 in liquid media

STRAIN	PLASMID AND PREVIOUS GROWTH TEMPERATURE	30°C	37°C	42°C
DOUBLING TIME IN MINUTES				
MM182	pND5(30°C)	160	48	100
dnaA5	pND5(37°C)	78->190	52	50->100
	pBR325(30°C)	64	64-> -	64-> -
MM185	pND5(30°C)	80	48	50
dnaA46	pND5(37°C)	50->100	45	48
	pBR325(30°C)	54	54->106	54-> -

A minus sign (-) indicates no growth.

For both MM185 (*dnaA46ts*) and MM182 (*dnaA5ts*) containing pND5 a reduced growth rate at 30°C compared to the control can be seen, the effect being much worse with MM182. Perhaps the most interesting feature of these growth curves is that they show the onset of cold sensitivity to be a gradual process following a shift down to 30°C from a higher temperature. A gradual reduction in growth rate is seen, taking several hours before it levels out at a new lower rate. Cultures pre-grown at 30°C grow at this reduced rate from the outset. This effect is not seen in a *dnaA*$^+$ control (MM18) containing pND5 (data not shown), indicating that it is not simply a consequence of a temperature shift down in conjunction with pND5. The effect is not seen following temperature shift down of a *dnaAts* mutant in the absence of pND5

FIGURE 4.2 Growth curves of MM185 pND5 and MM185 pBR325

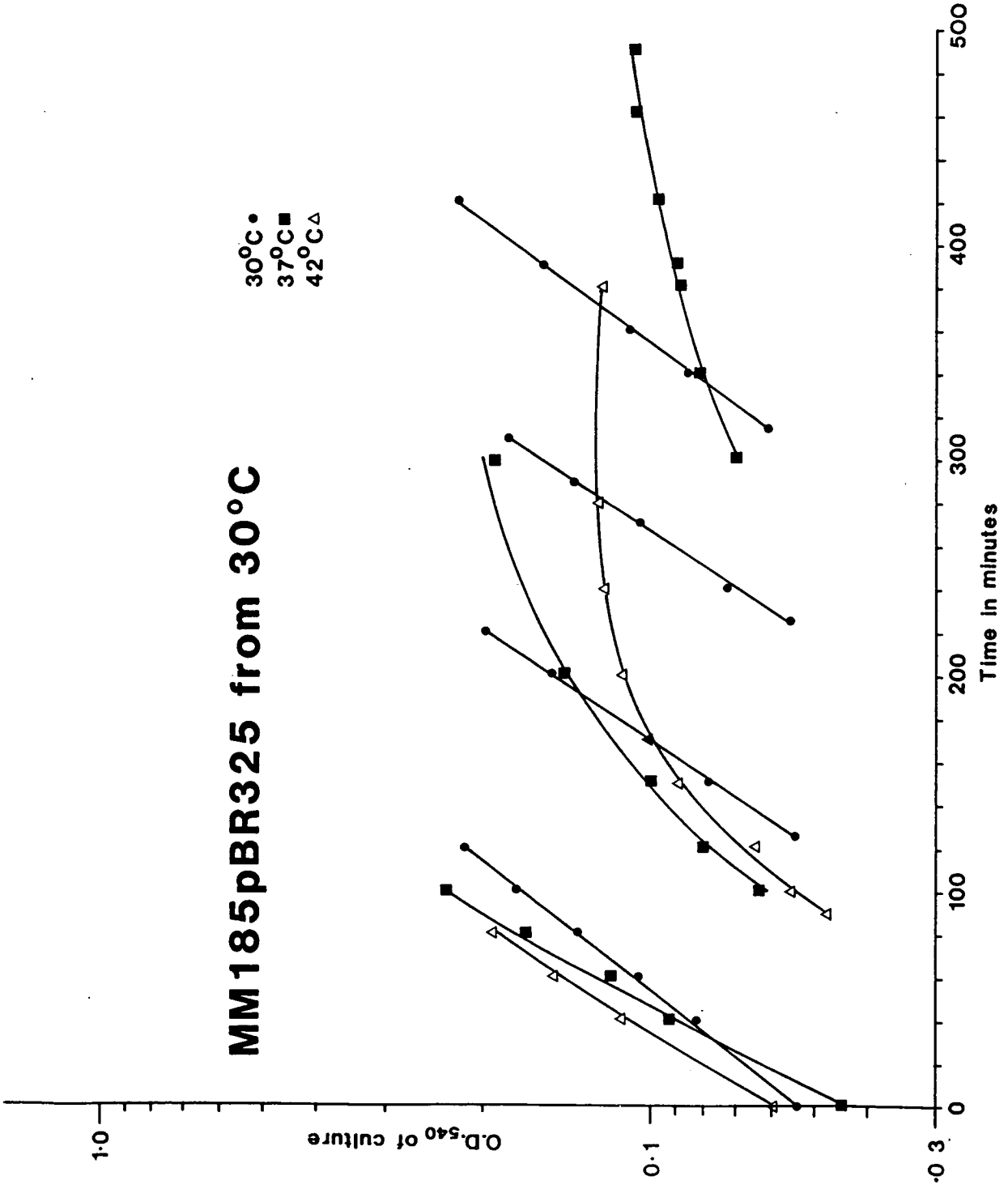


Figure 4.2: (continued)

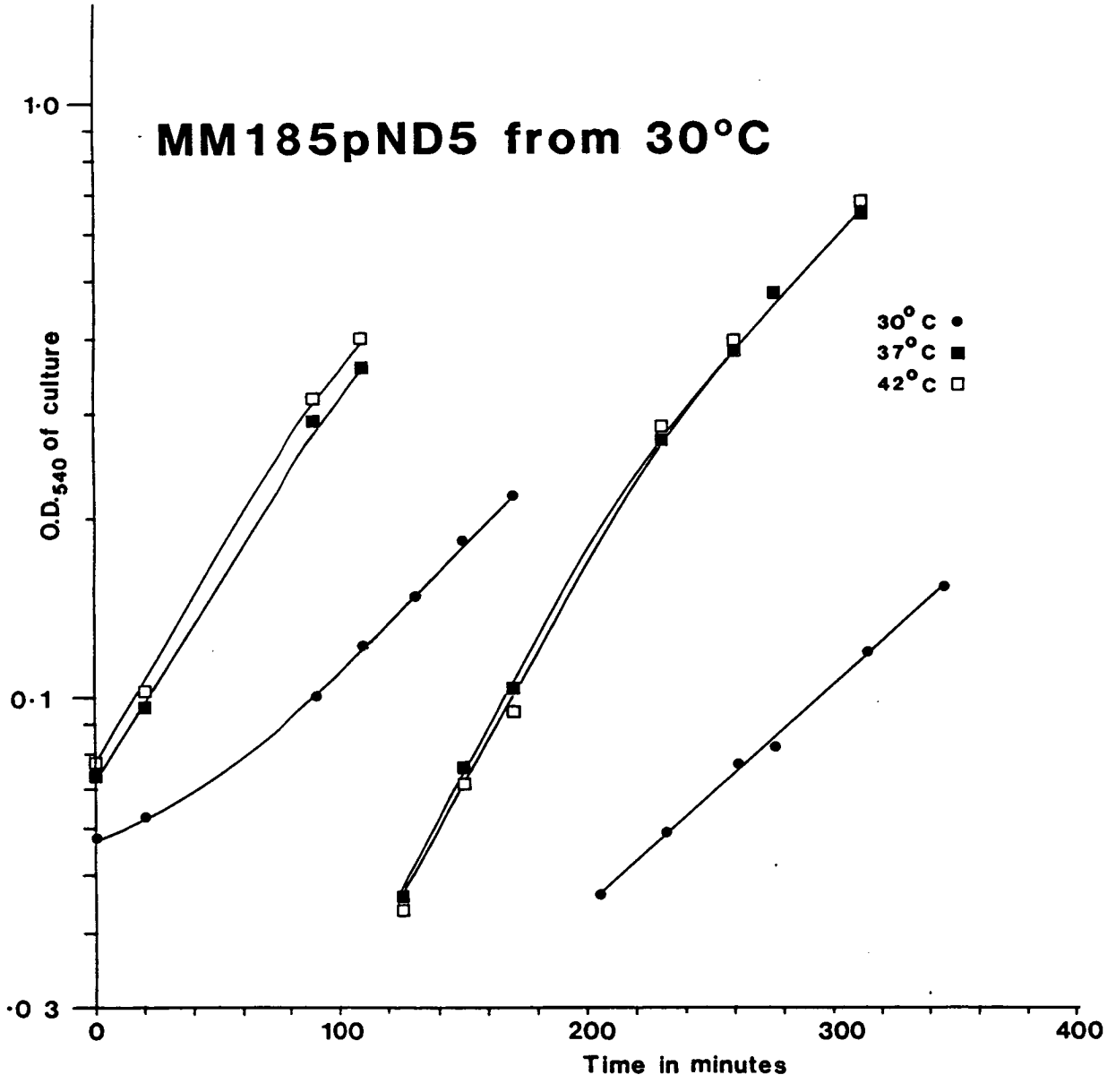


Figure 4.2: (continued)

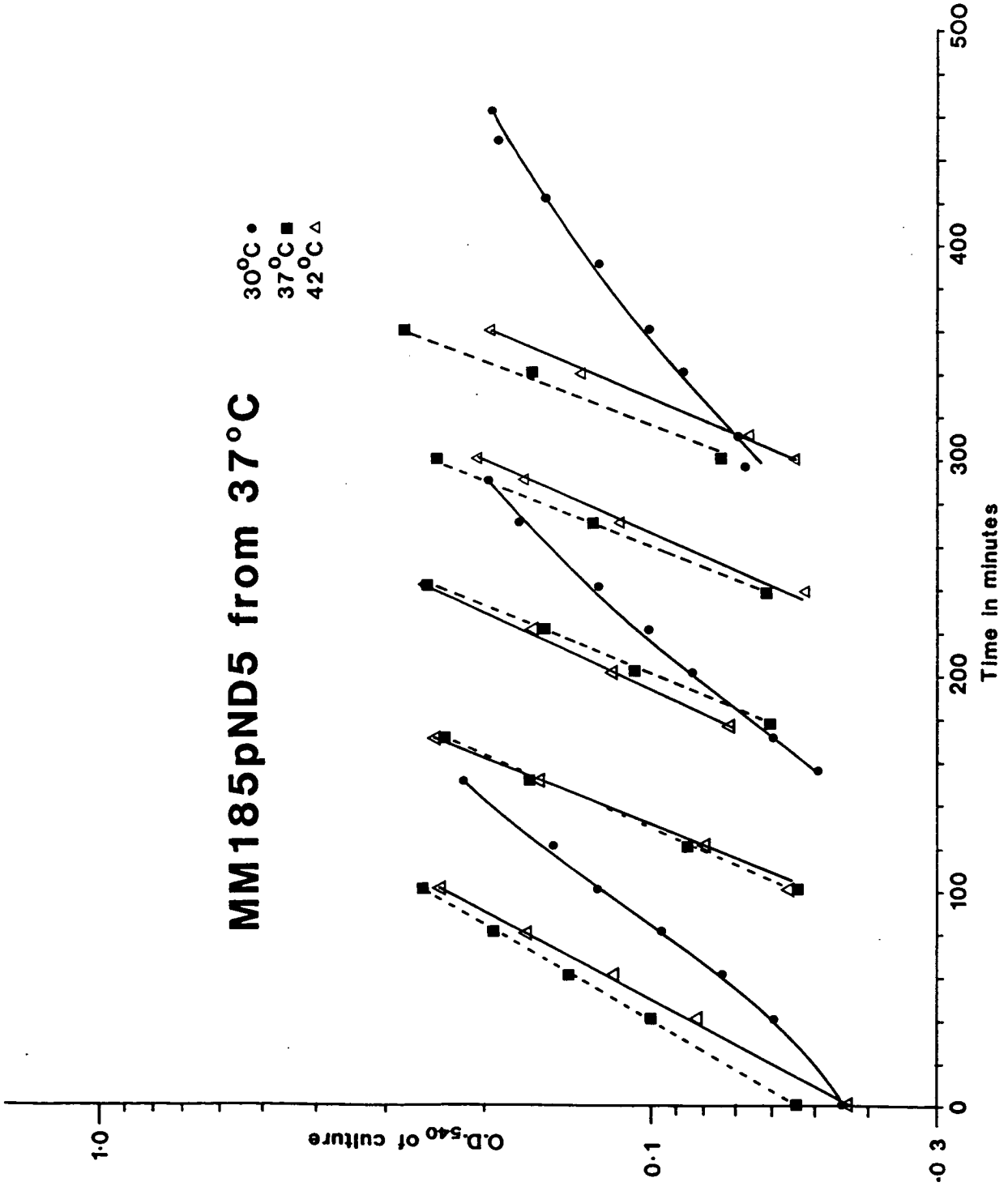


FIGURE 4.3 Growth curves of MM182 pND5 and MM182 pBR325

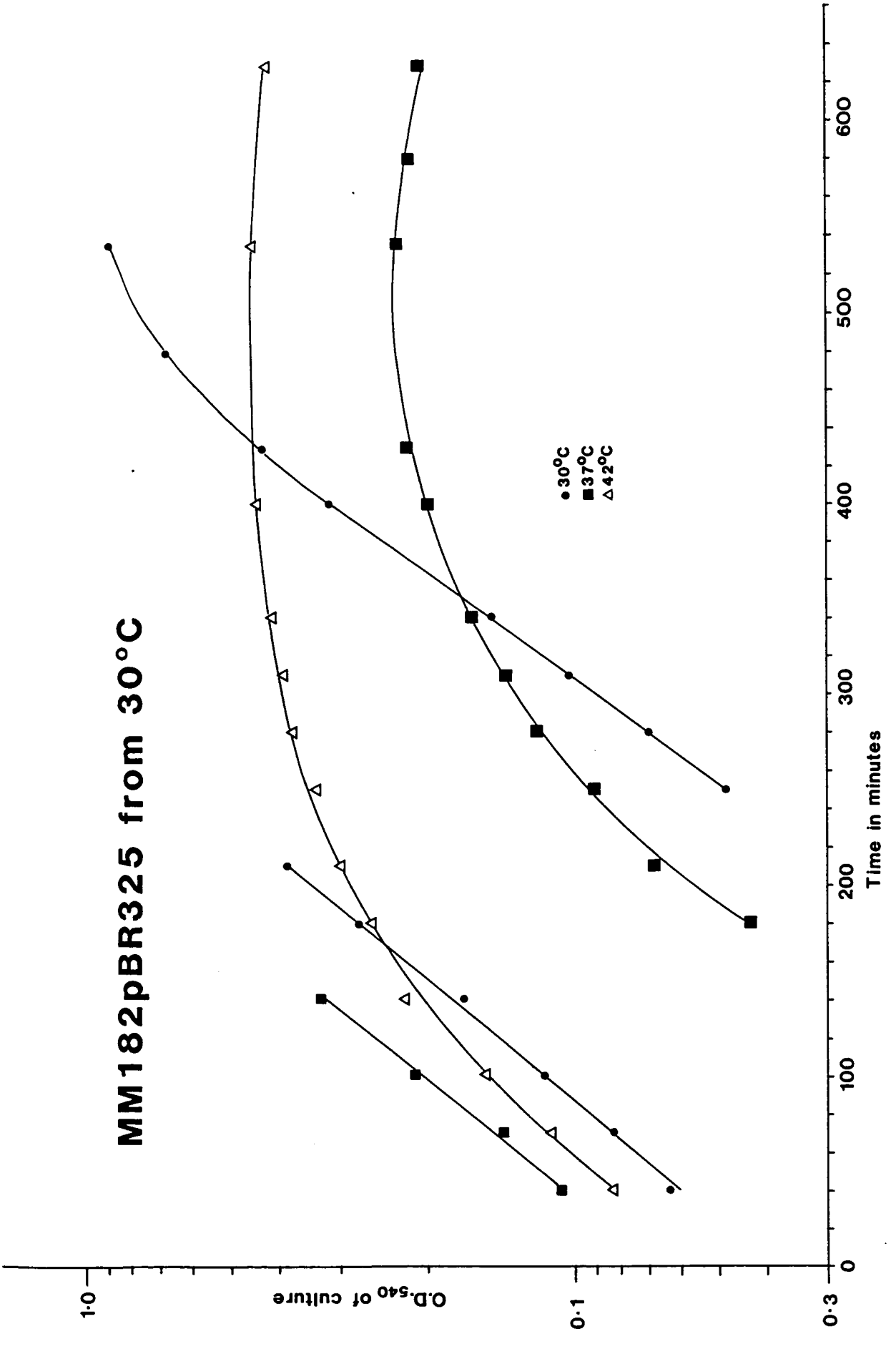


Figure 4.3: (continued)

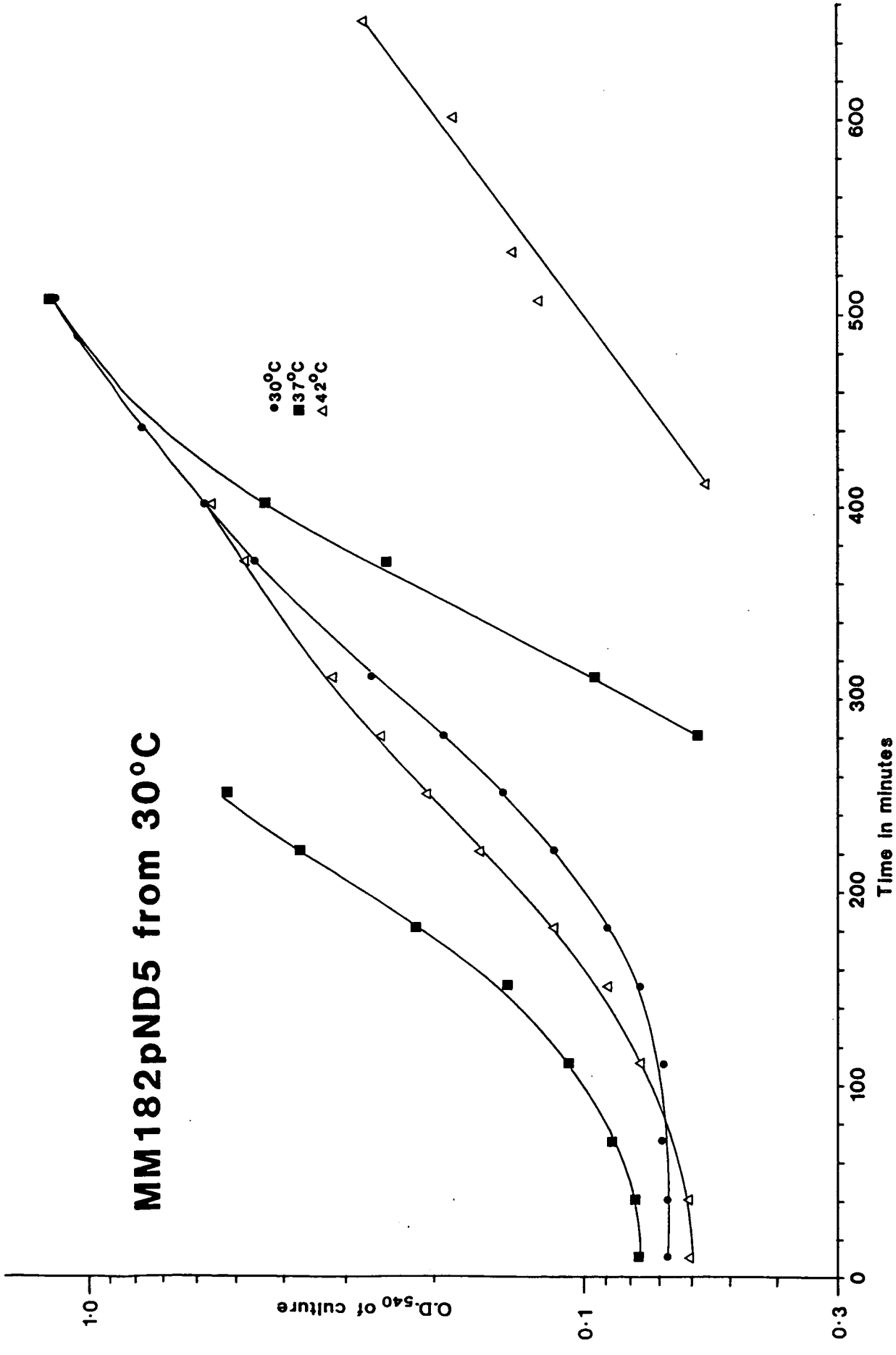
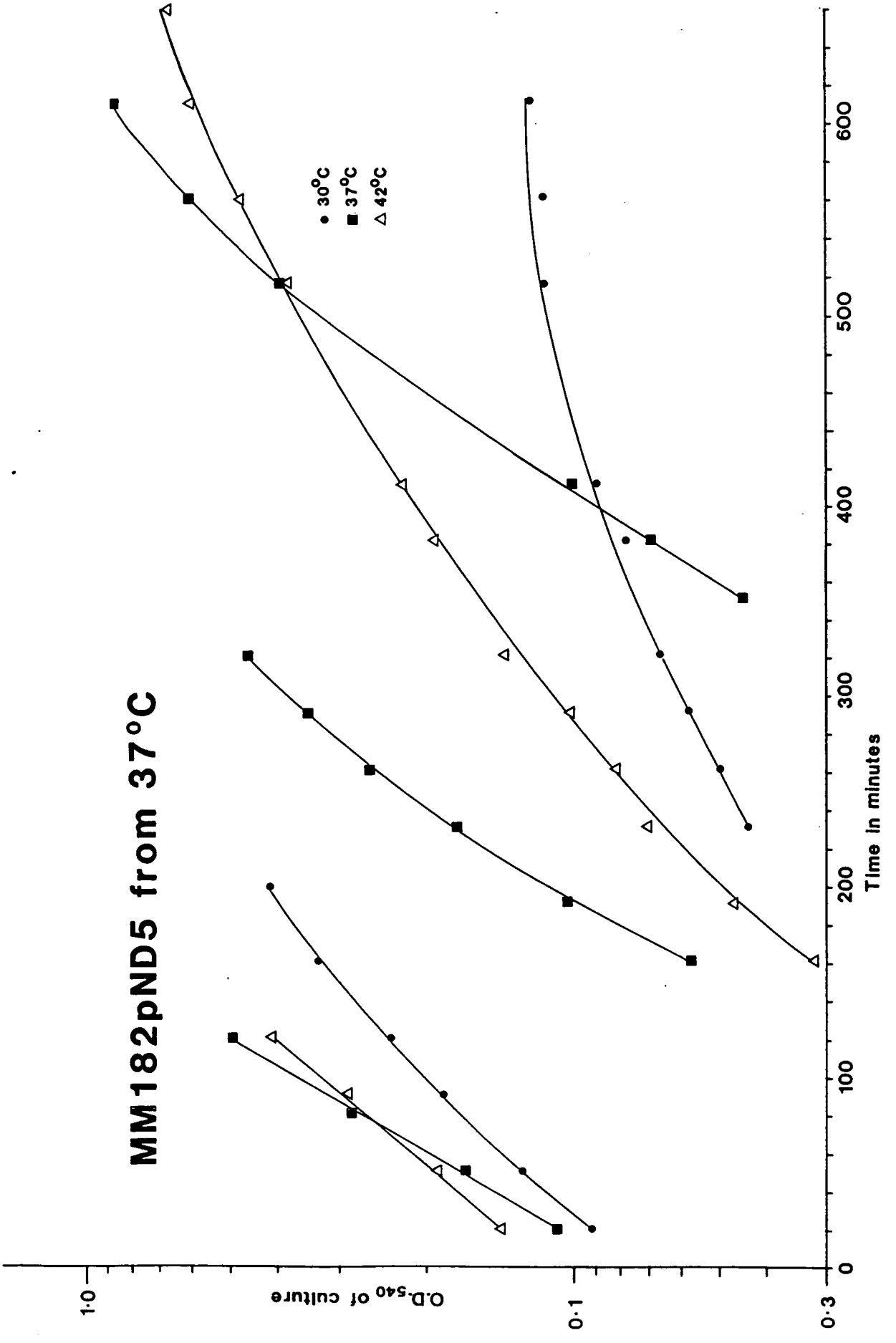


Figure 4.3: (continued)



(Hansen and Rasmussen 1977), indicating that it is due to a specific interaction between pND5 and the *dnaAts* allele. It would appear that some change in the cell involving the GroE and DnaAts proteins must be taking place in the temperature shift down, almost as if the slow accumulation of a 'cold sensitivity factor' is taking place. If cold sensitivity at 30°C involves some kind of 'adaption' (for example a reduction in the level of *groE* expression), it might be expected that following a shift from 30°C to 42°C, the growth rate would initially be reduced while this adaptation was reversed (i.e the level of expression was increased again). However, it appears that growth at 42°C accelerates to a new steady-state level immediately following a temperature shift from 30°C. That is, suppression is equally efficient whether the cells are in a state of 'cold sensitivity' or not, suggesting that the two mechanisms need not necessarily go hand in hand.

4.4.3 The degree of cold sensitivity appears to be correlated with the amount of GroE

Prolonged growth at 30°C results in isolates which no longer display cold sensitivity. The MM185 pND5 and MM182 pND5 cultures maintained at 30°C throughout the above experiment were streaked out on L-agar plus tetracycline (15µg ml^{-1}) at 30°C. Several large colonies were apparent against the general background of poor growth. These 'adapted' colonies are quite easy to isolate, and may be due to deletions from the plasmid or some other mechanism. One of these was selected for further study, and proved to be a chromosomal mutation affecting plasmid copy number (and consequently GroE levels), and is described further in chapter 7.

Several features of cold sensitivity suggest that the degree exhibited with any particular allele is due to the amount of GroE protein present (see table 4.8). Firstly, the copy number mutant of above exhibited lower GroE levels than the parental strain (figure 3.7), while at the same time not displaying the cold sensitivity of the parental. In addition, *dnaAts* strains lysogenised with λ*sidA* exhibit varying degrees of cold sensitivity when streaked at 30°C, presumably reflecting different levels of multiple lysogenisation (and hence GroE levels). A lysogen maintained at 40°C displays severe cold sensitivity when subsequently streaked at 30°C; conversely, a lysogen maintained at 30°C is not cold sensitive, but cannot grow when restreaked at 40°C. The former lysogen displays high levels of GroE protein; the latter exhibits only a very low level (figure 3.7), indicating a correlation between GroE levels and cold sensitivity. As has been previously mentioned (section 4.4.1), MM190 (*dnaA606*) is so cold sensitive that growth at 30°C and 37°C is not possible when transformed with pND5. However, a

TABLE 4.8 Cold sensitivity and GroE levels

	Growth at 30°C	Growth at 40°C
MM190 λ *sidA* (30°C)[a]	++	–
MM190 λ *sidA* (40°C)[b]	+	++
MM190 pND5	–	++
MM190 pJM32	+	++

a. Grown and maintained at 40°C.
b. Grown and maintained at 30°C.

λ *sidA* lysogen when grown at 42°C and transferred to 30°C can grow to a certain extent, revealing a heterogeneity of colony size presumably reflecting the copy number of the lysogenic phage (less copies of the phage are probably present than copies of the plasmid). Since this culture had been pre-grown at 42°C, it would suggest that suppression is possible with lower levels of GroE than would cause cold sensitivity. This is further suggested by an examination of the behaviour of strains MM185 (*dnaA46ts*) and MM190 (*dnaA606ts*) when transformed with pJM32. This plasmid (described in chapter 3) carries a *groEL-tet* gene fusion as well as the intact *groES* gene, and is able to suppress both *dnaA46ts* (MM185) and *dnaA606ts* (MM190), despite the fact that it is maintained in a low copy number. However, when streaked at 30°C, very little cold sensitivity is apparent (table 4.8), possibly reflecting the lower GroE protein levels. It is possible that the change to the *groEL* gene in pJM32 has reduced its capacity to cause cold sensitivity while increasing its ability to suppress. However, this experiment does suggest that one or both of the *groE* genes is resposible for cold sensitivity, since both of the other insert encoded proteins of pND5 are absent from pJM32 (figure 3.2).

4.4.4 Transformation of *oriC* plasmids into *dnaAts* strains: correlation with cold sensitivity

What are the causes and results of cold sensitivity? A possible clue is provided by the behaviour of *dnaAts* strains when transformed with *oriC* replicons.

The original reason for examining the phenotype of *dnaAts* strains when transformed with *oriC* replicons was to see if they might be able to 'titrate out' the

suppressing effect of excess *GroE*. If suppression occurred by increasing the amount or effectiveness of the mutant DnaA protein, the presence of additional origins might well have an adverse effect on the ability of *groE* to mediate suppression.

To investigate this possibility, the *oriC* plasmid pHP6 was used. This plasmid contains the chloramphenicol and tetracyline resistance genes of pBR325 (Bolivar 1978) and the *oriC* region of pCM959 (Meijer *et al.* 1979). It was used to transform strain MM185 (*dnaA46ts*) pND5 at 30°C and 40°C, selecting for chloramphenicol resistance (pHP6) and ampicillin resistance (pND5). As a control to compare growth, a dilution of competent cells containing pND5 only was plated on ampicillin at 30°C and 40°C. The results of this transformation are shown in table 4.9. At 40°C it would appear that the presence of the *oriC* plasmid is certainly hindering suppression by pND5, in keeping with the above hypothesis. However, at 30°C growth is also markedly reduced, when suppression of the *dnaAts* phenotype should not be necessary. These colonies require at least two days at 30°C to become visible to the naked eye, indicating that the pHp6-pND5 combination at 30°C causes an even more severe reduction in growth than pND5 alone (i.e. the normal cold sensitive phenotype).

TABLE 4.9 Effect of the *oriC* plasmid pHP6 on the growth of MM185 pND5

	30°C	40°C
COLONY DIAMETER		
MM185 (pND5) pHP6[a]	<<0.1mm[b]	<0.1mm
MM185 (pND5)	0.1-0.3mm	1.5mm

a. Selected on amp chl plates, MM181 pND5 grown on amp alone.

b. Plates were incubated for 48hrs before colonies became visible. All other plates were incubated overnight (c. 18hrs) before colony sizes were measured.

This initial result suggested that not only was pHP6 reducing the ability of pND5 to mediate suppression of *dnaAts*, but also that it was increasing the cold sensitive phenotype of the plasmid-bearing strain at 30°C. To investigate this, strain MM181 (*dnaA167ts*) pND5 was transformed with pHP6 as above. *dnaA167ts* is suppressable by *groE* over-expression but does not exhibit cold sensitivity in conjunction with pND5 at 30°C. The results are shown in table 4.10. Double

transformants do not exhibit cold sensitivity at 30°C; nor does the presence of pHP6 effect the ability of pND5 to mediate suppression at 40°C. Similar results were obtained when λ *sidA* lysogens of MM181 (*dnaA167ts*) and MM185 (*dnaA46ts*) were transformed with pHP6 (data not shown).

TABLE 4.10 Effect of the *oriC* plasmid pHP6 on the growth of MM181 pND5

	30°C	40°C
COLONY DIAMETER		
MM181 (pND5) pHP6[a]	1.0mm	1.5mm
MM181 (pND5)	1.0mm	1.5mm

a. Selected on amp chl plates, MM181 pNDS grown on amp alone. All plates were incubated overnight (c. 18hrs) before colony sizes were measured.

The original experiment examining growth of MM185 pND5 pHP6 transformants at 40°C suggested that extra origins did indeed reduce the ability of *groE* to suppress. However, the reduced growth at 30°C, and the absence of any effect in MM181 at either temperature suggested that the extra origins were not affecting supression specifically, but were causing a more general reduction in growth. Furthermore, the absence of any reduction in growth of MM181 (*dnaA167ts*) suggested that the effect might be allele specific. Accordingly, it was decided to transform strains MM181–MM190 with pHP6. This would indicate whether or not the presence of the *oriC* plasmid alone would be enough to cause a reduction in growth rate. Additionally, it would furnish information about any allele specificity of the effect. Transformants were plated at 30°C under chloramphenicol (5μg ml^{-1}) selection, and plasmid pBR325 was included as a control to measure transformation efficiency and allow a comparison of growth rates. The figures shown in table 4.11 represent the number of colonies obtained with 20ng of DNA.

These results show that both of the above hypotheses are indeed correct. pHP6 alone is enough to cause a reduction in growth rate. Also, the effect is allele specific; strains MM181 (*dnaA167ts*), MM183 (*dnaA204ts*), and MM186 (*dnaA203ts*) do not exhibit this effect. In addition, the degree is allele specific; strains MM187–MM190

TABLE 4.11 Effect of *oriC* plasmid pHP6 on growth of isogenic *dnaAts* series MM181-190

STRAIN	MUTATION	PLASMID USED FOR TRANSFORMATION[a] pBR325		pHP6	
NO. OF TRANSFORMANTS AND COLONY DIAMETER					
MM181	*dnaA167*	≈200	1.0mm	≈2000	1.0mm
MM182	*dnaA5*	≈200	1.0mm	≈600	<0.1mm[b]
MM183	*dnaA204*	≈300	1.0mm	≈2000	1.0mm
MM184	*dnaA508*	≈200	1.0mm	≈2000	<0.1mm[b]
MM185	*dnaA46*	≈200	1.0mm	≈2000	<0.1mm[b]
MM186	*dnaA203*	≈200	1.0mm	≈1000	1.0mm
MM187	*dnaA602*	≈100	1.0mm	-	-
MM188	*dnaA601*	≈100	1.0mm	-	-
MM189	*dnaA604*	≈100	1.0mm	-	-
MM190	*dnaA606*	≈100	1.0mm	-	-

a. 20ng of plasmid DNA used, selecting for chl^R. Shown are the approximate no. of transformants obtained together with the measured colony diameter. No pHP6 transformants were obtained for strains MM187-190.

b. These plated were incubated for c.48hrs before colonies became visible. All other plates incubated for c.18hrs (overnight). Plates incubated at 30°C.

could not be transformed with this plasmid although transformation with pBR325 was possible.

Perhaps the most interesting aspect of this '*oriC*' sensitivity is when the pattern of allele specificity is compared to that of cold sensitivity caused by over expression of *groE* (figure 4.4). With the exception of *dnaA508ts*, which is *oriC* sensitive but not cold sensitive, the pattern is identical, even down to the degree of the effect. For example, strains MM187-MM190 (*dnaA602ts, dnaA601ts, dnaA604ts, dnaA606ts*) are so cold sensitive they are unable to grow with pND5 at 30°C; similarly they are so *oriC* sensitive that transformation with pHP6 is not possible. This suggests that the mechanism responsible may be the same in both cases. Furthermore, although these *dnaAts* strains can all grow at 30°C, those which exhibit *oriC* sensitivity and cold sensitivity obviously possess a mutant DnaA protein even at a temperature permissive for growth.

FIGURE 4.4 Correlation between cold sensitivity and *oriC* sensitivity

	dnaAts allele no. (location with respect to promoter of *dnaA*)									
	← *dnaA*									P
	203	*204*	*602*	*601*	*604*	*606*	*5*	*46*	*167*	*508*
cold sens.[a]	–	–	+++	+++	+++	+++	+	+	–	–
oriC sens.[b]	–	–	+++	+++	+++	+++	+	+	–	+
groE supp.[c]	–	–	+	+	+	++	++	++	++	–

a. Cold sensitivity caused by over-expression of groE. – = No cold sensitivity; + = cold sensitivity at 30°C; +++ = cold sensitivity so severe that no growth is possible at 30°C.

b. Reduced growth due to presence of oriC plasmid pHP6. – = No effect; + = reduced growth; +++ = combination inviable for growth.

c. Suppression by over-expression of groE shown for comparison. + = suppression up to 40°C only; ++ = suppression up to 42°C.

4.4.5 Fluorometric assay of DNA in *dnaAts* strains

If cold sensitivity and *oriC* sensitivity are due to the same mechanism, this would suggest that the former condition is probably characterised by too little DnaA per origin, since adding extra origins is effectively increasing the *oriC* to DnaA ratio. Since DnaA has been postulated to play some kind of positive regulatory role in the initiation process (e.g. Atlung *et al.* 1987), such a condition could result in under-initiation. Extra origins would presumably compete with the chromosomal *oriC* for the small amount of DnaA activity present, thereby causing reduced DNA replication. Obviously such a condition would be expected to be deleterious to the cell.

What if cold sensitivity is due to over-initiation? A precedent for this already exists in fact, with an allele of *dnaA*, called *dnaAcos* (Kellenberger-Gujer *et al.* 1978.). This is a mutant which was originally selected as a revertant of the temperature sensitive phenotype of a *dnaA46ts* mutation, but which in fact turned out to be due to intragenic suppression (i.e. a double mutant). Although this double mutant was now able to grow at 42°C, when returned to 30°C it exhibited a marked reduction in growth rate (i.e. it exhibited cold sensitivity). A feature of this cold sensitivity is that over-initiation of DNA replication occurs. Subsequent work by Braun *et al.* (1987) has suggested that this is due to an increased activity of the mutant form of the protein, although it is not clear whether over-initiation is a cause or an effect of cold

sensitivity. If suppression by *groE* is due to an increase in the activity of the DnaA46ts protein (for example by increasing the efficiency of binding to DNA), this could explain both the suppression and cold sensitivity phenotypes. If so, an increase in the DNA content of such cold sensitive strains should be seen. It should be noted however that this theory does not tie in with the '*oriC* sensitivity' described above, in which too little DnaA activity is presumed to be the cause of the reduced growth.

As an investigation into this, it was decided to measure the DNA in MM18 (*dnaA*$^+$) and MM185 (*dnaA46ts*) containing pND5 or pBR325 (as a control) by fluorometric assay. Strains were grown at both 30°C and 42°C and the DNA content measured. When transformed into MM185, pND5 causes cold sensitivity, so an examination of the DNA content in such a strain should hopefully give some insights into the nature of this. In addition, comparison with MM18 should give some indications into the efficiency of suppression by *groE*. To conduct the experiment, MM18 pND5, MM18 pBR325 and MM185 pND5 were grown at 37°C until mid log phase, then diluted back at 30°C or 42°C and grown for several hours until steady state growth was achieved, whereupon samples for DNA measurement were taken. MM185 pBR325 was grown at 30°C until steady state, then diluted back at 30°C or 40°C and samples taken immediately. Growth was followed and samples taken every 30 minutes over the next 3 hours. The results are shown as specific DNA content (Total DNA/Optical Density of Culture) in table 4.12. In the *dnaA*$^+$ strain the presence of pND5 appears to make no difference to DNA content, either at 30°C or 42°C. In MM185 (*dnaA46ts*) DNA content is slightly reduced at 30°C, indicating that although growth is possible at this temperature the DnaA46ts protein is not fully functional. At 42°C DNA content is very much reduced, as would be expected. Examining MM185 pND5 at 30°C it would appear that DNA content has been increased to wild type levels compared with MM185 pBR325 at this temperature. As such, cold sensitivity does not appear to be correlated with under or over-initiation on a gross scale. The cold sensitivity of pND5-containing strains is thus quite different from that of the *dnaAcos* mutant, in which a doubling of the cellular DNA content is seen (Kellenberger-Gujer *et al.* 1978). Although no major changes in the cellular DNA content are seen, it is possible that over-initiation (with a subsequent rapid termination of these forks) is occurring, similar to that described following over-expression of the *dnaA* gene (Atlung *et al.* 1985b, 1987). This would not be detected by a measurement of total cellular DNA, and would probably require the measurement of the origin to terminus marker ratio by means of radioactive probes. Interestingly, suppression by *groE* does not appear to be perfect at 42°C; the overall DNA content is less than in a *dnaA*$^+$ strain, although considerably more than is present in a non suppressed culture.

TABLE 4.12 Effect of the *groE* plasmid pND5 on cellular DNA

STRAIN	PLASMID	30°C	42°C
DNA CONTENT			
MM185 *dnaA46*	pBR325	916 ± 25	369 ± 51
	pND5	1199 ± 54	990 ± 108
MM18 *dnaA*$^+$	pBR325	1061 ± 55	1130 ± 92
	pND5	1152 ± 8	1116 ± 73

Shown is the specific DNA content (i.e. DNA per O.D. equivalent) together with the standard deviation in arbitary units.

4.4.6 Is cold sensitivity present in *oriC* delete strains?

The DNA measurements described above suggest that cold sensitivity does not cause (or is not a result of) any large scale effect on DNA replication. But why should the allele specificity of cold sensitivity be so similar to *oriC* sensitivity? Is cold sensitivity due to a lack of DnaA as would be suggested by the *oriC* results, or is the similarity due to some other factor?

If cold sensitivity is due to an interaction at *oriC*, then it should not be possible to demonstrate it in an *oriC* delete strain. If cold sensitivity can be caused in an *oriC* delete strain, this would suggest that cold sensitivity and suppression are not a direct consequence of suppression of *dnaAts* mutations by *groE*.

To examine this, pND5 was transformed into strain TPK30. This is *dnaA46ts* integratively suppressed with the non-DnaA requiring replicon pKN500. It is *oriC*$^+$, so it can replicate from either *oriC* or the pKN500 origin at 30°C. Plasmid pND5 was also transformed into DK201, which is both *dnaA5ts* and *oriC* delete. The strain is mutant in RNAaseH (*sdrA*) and so is able to replicate from secondary origins located around the chromosome. As this strain is rich medium sensitive it was maintained on minimal media throughout. As a control, both strains were also transformed with pBR328, and the number of transformants and the colony size noted at both 30°C and 37°C. Results are shown in table 4.13. It is clear that pND5 is causing cold sensitivity in TPK30. This could either mean that replication in this strain is now occurring from *oriC*

TABLE 4.13 Transformation of strains TPK30 and DK201 with pND5

STRAIN	PLASMID	30°C		37°C	
NO. TRANSFORMANTS AND COLONY DIAMETER (MM)					
TPK30[a]	pBR328	≈200	1.0	≈200	1.5
	pND5	≈1200	<0.1	≈1200	1.5
DK201[b]	pBR328	≈500	0.5-1.2	≈500	1.5
	pND5	2	0.2	≈150	0.2-0.7

a. Grown on LA plates with ampicillin selection (c.18hrs growth).

b. Grown on minimal plates with ampicillin selection (c.48hrs growth).

instead of the pKN500 origin, and that cold sensitivity is due to a replication dependent interaction at *oriC* (i.e. a dominant effect), or else replication is proceeding from the pKN500 origin and cold sensitivity is due to something else. Results with DK201 are similarly inconclusive. The much reduced transformation frequency at 30°C suggests that perhaps cold sensitivity is taking place in the absence of *oriC*; however, although viability is reduced compared to 37°C, the growth rate reduction (compared to a pBR328 control) appears to be roughly the same whether at 30°C or 37°C. Because of this, it is difficult to determine if this is genuine 'cold-sensitivity' or whether pND5 has a more general deleterious effect on the cell. It could be that the deletion of *oriC* means that 'cold sensitivity' is now apparent at higher temperatures than when *oriC* is present. However, on balance, it does perhaps suggest that the specific deleterious interaction between *groE* and certain *dnaAts* alleles is not restricted to an effect involving *oriC* (if indeed cold sensitivity involves *oriC* under any conditions).

4.4.7 Summary and discussion of cold sensitivity

The subject of cold sensitivity is difficult to present in a concise and unambigious manner. If cold sensitivity is a direct consequence of suppression by over-expression of *groE* then it may provide important insights into the mechanisms involved. Alternatively, it may simply be the result of an interaction essentially unconnected with suppression. A summary of the main features of the cold sensitivity caused by over-expression of *groE* in conjunction with *dnaAts* is presented below.

- Cold sensitivity is allele specific, both in degree and occurrence.
- Cold sensitivity takes time to appear following a shift-down from a higher temperature.
- The degree of cold sensitivity depends on the amount of *GroE*: smaller quantities are able to suppress than appear to cause cold sensitivity.
- The pattern of *oriC*-plasmid sensitivity of some *dnaAts* alleles is very similar to the allele specificity of cold sensitivity.
- DNA replication (on a gross scale) is not affected.
- The effect appears to be (at least partially) *oriC* independent.

In addition to these features, a mention of the cold sensitivity present in *dnaA*$^+$/*dnaAts* should be made prior to a discussion of the phenomena. Merodiploids between most *dnaA*$^+$/*dnaAts* alleles are also cold sensitive (Hansen *et al.* 1984), presenting a pattern of allele specificity similar to that of cold sensitivity and *oriC* sensitivity. If suppression by *groE* is informational in nature (analogous to tRNA suppressors for example), a consequence would be the production of both mutant and non-mutant forms of the DnaA protein. This would explain why suppression by *groE* leads to cold sensitivity, and also why the pattern of cold sensitivity matches that of *dnaA*$^+$/*dnaAts* merodiploids (figure 4.5). For reasons which will be discussed later (section 4.7) it appears unlikely that informational suppression is taking place; however, the similar pattern of allele specificity is perhaps significant, and provides another addition to an already confused picture.

At this juncture it is possibly best to examine the overall phenomenon of cold sensitivity as comprising two basic questions:-

1. Are the *oriC* effect, the cold sensitivity of *groE* over-expression, and the cold sensitivity of *dnaA*$^+$/*dnaAts* merodiploids due to the same mechanism?
2. Is cold sensitivity an integral part of the suppression process (for example a consequence of increased activity of the suppressed DnaAts protein).

FIGURE 4.5 Cold sensitivity of *dnaA*$^+$/*dnaAts* merodiploids

	dnaAts allele no. (location with respect to promoter of *dnaA*)									
	← *dnaA*									P
	203	*204*	*602*	*601*	*604*	*606*	*5*	*46*	*167*	*508*
cold sens.[a]	–	–	+	+	+	+	+	+	–	–
oriC sens.[b]	–	–	+	+	+	+	+	+	–	+
dnaA$^+$/*dnaAts*[c]	–	–	+	+	+	+	+	+	–	–

a. Cold sensitivity caused by over-expression of groE.

b. Sensitivity to oriC plasmids

c. Cold sensitivity of dnaA$^+$/dnaAts merodiploids (data from Hansen et al. 1984).

Firstly, is the reduced growth due to the presence of *oriC* plasmids, the cold sensitivity due to over-expression of *GroE*, and the cold sensitivity of *dnaA*$^+$/*dnaAts* merodiploids due to the same mechanism, as the similar pattern of allele specificity would suggest (figure 4.5)? Or are the mechanisms involved different in each case, and the only similarities (coincidental or otherwise) being in the pattern of allele specificity exhibited? Whilst it is difficult to conceive of an all-embracing mechanism which could explain the three phenotypes, certain characteristics of each could be explained by a similar mechanism. For example, if *groE* acted as an informational suppressor this could explain the similar pattern of allele specificity between *groE* and merodiploid cold sensitivity. Alternatively, since extra copies of *oriC* have been reported (Hansen *et al.* 1987) to 'titrate out' the DnaA present in the cell (and thereby increase *dnaA* expression), it is likely that the addition of extra origins is resulting in increased production of the mutant DnaAts protein. The presence of increased amounts of mutant DnaA protein might well result in cold sensitivity. This would tie in with a model for *groE* suppression where it acted by increasing the amount of the mutant DnaAts protein, either by increasing its expression, or by protecting the protein from degradation for example. The extra DnaAts protein would be presumed to cause cold sensitivity by some unspecified action in the cell. Thus extra *oriC* would be indirectly responsible for cold sensitivity; this would fit in with cold sensitivity being possible in an *oriC* delete strain. Under this model then, cold sensitivity is not due to a direct interaction between *oriC* and the DnaAts protein.

This latter model also brings us conveniently onto the second question,

namely, is cold sensitivity an integral part of the suppression process? (Both of them being due to the same consequence of GroE action; for example, increasing the binding of the DnaA protein to DNA causes both cold sensitivity *and* suppression). Evidence that it is can be summarised as follows:- (1) The similar pattern of allele specificity between cold sensitivity and *oriC* sensitivity suggests that cold sensitivity may be due to some interaction at *oriC*. (2) The degree of the effect is also allele specific; again the patterns are very similar. (3) Perhaps the strongest evidence that cold sensitivity is a consequence of suppression is provided by a more detailed investigation of the allele specificity of the effect. All suppressable *dnaAts* alleles (with the exception of *dnaA167ts*) are also cold sensitive. Similarly, those alleles which are not suppressed (without exception) do not exhibit cold sensitivity, suggesting that suppression and cold sensitivity go hand in hand. (4) Using an *in vitro* replication system it has been shown that the formation of the DnaA-*oriC* initiation complex is a cold sensitive event (van der Ende *et al.* 1985). While an *in vitro* system need not necessarily present an accurate portrayal of the actual situation *in vivo*, it does provide a possible indication of how *groE* mediated cold sensitivity might occur. At least in some circumstances, it would seem that cold sensitivity can be correlated to events occurring at *oriC*.

However, perhaps this is a misleading picture. It may be more accurate to ask the question 'What features of those alleles make them suppressable and liable to be cold sensitive?' rather than assume that the one is necessarily the consequence of the other. In fact a considerable quantity of evidence suggests that cold sensitivity is not a direct consequence of suppression of *dnaAts* alleles by over-expression of *groE*. (1) DNA content appears to be normal in a cold sensitive strain. Since suppression affects the ability of DnaAts to act at the initiation of DNA replication, it would be expected that if cold sensitivity was a direct consequence of the suppression process then DNA replication might be affected in some manner. (2) The fact that cold sensitivity appears to be an adaptive process whereas suppression is not. Strains pre-grown at 30°C are cold sensitive and able to grow at 42°C, while strains grown at 42°C are not immediately cold sensitive following a shift down to 30°C. This demonstrates a separation of the cold sensitive and suppression effects of excess GroE. (3) Strains transformed with pJM32 can be suppressed by the mutant GroEL protein it encodes but cold sensitivity seems to be much reduced compared to pND5. (4) The results with the *oriC* delete strain DK201 suggest that cold sensitivity is at least partially present in strains where suppression does not occur. (5) The result using the *dnaA508ts* allele, which is *oriC* sensitive but not cold sensitive, suggesting that the mechanisms involved, at least in this instance, are different. (6) The identical

pattern of allele specificity between *groE* and *rpoB* suppression of *dnaAts* (section 4.3.3) suggests that a similar mechanism is involved. However, *rpoB* suppressors of *dnaAts* do not cause cold sensitivity (Atlung 1984), suggesting that the processes of suppression and cold sensitivity can be separated. (7) It is possible to isolate mutants which are no longer cold sensitive but which are suppressable by *groE* at 42°C. Extended growth of cold sensitive strains at 30°C results in the isolation of faster growing derivatives. While some of these have lost the ability to grow when transferred back to 42°, (possibly due to a reduction in *groE* over-expression for example), others had retained the ability to grow at this temperature, indicating at least a partial separation of the cold sensitive and suppression phenotypes of over-expression of *groE* (data not shown).

Examination of the above data should make it clear to the reader that the question of whether or not cold sensitivity is an integral part of the suppression process is a difficult one to answer, and I at least will not attempt to do so here. With an answer in either direction being justifiable on several counts it is perhaps best to return a verdict of 'not proven' in this instance. It may be, for example, that both hypotheses are partially correct, and the all embracing term of 'cold sensitivity' is due to a mixture of interactions at *oriC* and elsewhere in the cell, either of which can be separated and visualised as cold sensitivity on its own. Room for considerable speculation exists, on a variety of fundamental questions. For example, what actually is cold sensitivity? Whether it is due to over-production of a mutant form of the DnaAts protein, or the presence of *dnaA*$^{+}$/*dnaAts* merodiploids, or indeed some other mechanism, why should this have an adverse affect on cellular growth? Why is it allele specific? Why does it take time to appear following a shift down in growth temperature? These are all interesting questions in their own right, as well as in the more general context of an investigation into the mechanistic process of suppression of *dnaAts* by over-expression of *groE*.

4.5 Expression from the *dnaA* promoter in strains over-expressing *GroE*

As has been discussed in section 4.3, the pattern of allele specificity exhibited by *groE* suppression of *dnaAts* is identical to that seen with certain *rpoB* mutants. This could be due to interactive suppression between GroE, RNA polymerase and DnaAts, or alternatively, both types of suppression could be due to higher levels of the mutant DnaAts protein as a result of increased expression. As an investigation into whether *groE* over-expression lead to increased expression of the *dnaA* gene, it was decided to measure transcription originating from the *dnaA* promoter by means of

fusions to the *lacZ* gene of *E.coli*. The product of the *lacZ* gene is the enzyme β-galactosidase, whose activity can easily be be measured by means of a colourimetric assay (Miller 1972). By use of this system, transcription originating from the *dnaA* promoter can be estimated from the β-galactosidase activity present in a cell extract. As well as providing insights into the mechanism of suppression, such an approach might also give pointers towards any changes in *dnaA* expression under conditions of cold sensitivity.

The use of translational fusions involving the promoter region of *dnaA* fused to the *lacZ* gene have been reported before (Braun *et al.* 1985, Atlung *et al.* 1985a). While not as satisfactory as a direct measurement of the mRNA levels of the gene in question, such fusions are useful in presenting a general picture of the level of transcription originating from the *dnaA* promoter. Using such a system it has been shown that *dnaA* expression is autoregulatory in nature; transcription (i.e. β-galactosidase activity) increases upon thermal inactivation of the DnaAts protein. Similarly, increasing the amount of DnaA protein present in the cell (by over expression from the *lac*UV5 promoter) results in a decrease in expression (Braun *et al.* 1985). If *groE* suppression were mediated by increasing transcription of the *dnaAts* gene, considerably more expression than is seen following thermal inactivation of the DnaAts protein would be expected to occur in suppressed strains then. Use of this system should give indications as to whether suppression of *dnaAts* by *groE* is due to increased transcription of the mutant gene. In addition, if cold sensitivity is due to reduced or increased amounts of the DnaAts protein at 30°C, this might also become apparent upon such examination.

Lysogens of the recombinant lambda phage λRB1 (Braun *et al.* 1985) were used in the experiments described herein. This phage contains the promoter region of *dnaA* fused to β-galactosidase. Cells lysogenic for λRB1 carry a single copy of the *dnaA-lacZ* fusion located at the λ attachment site on the *E.coli* chromosome in addition to the chromosomal copy of the mutant or wild type *dnaA* gene.

4.5.1 Transcription from P*dnaA* in a *dnaA*$^+$ strain

Initially it was decided to investigate the effect of over-expression of *groE* on expression from P*dnaA* in a *dnaA*$^+$ strain. Strain TP91 (*lac* delete, *dnaA*$^+$, λRB1 lysogen) was used for this purpose. It was transformed with pND5 and pBR325 (as a control), and transformants were screened by plasmid preparation and restriction analysis. The growth rates of both types of transformant were similar, with perhaps a slight reduction seen in the pND5-containing strain (table 4.14). To perform the assay,

fresh overnight cultures were diluted 1:100 the following day into L-broth containing tetracycline (15µg ml^{-1}) and grown for 3-4 hours before sampling was begun. Dilutions were periodically made such that the $O.D._{540}$ was maintained between 0.06-0.20 and steady-state growth was achieved. To allow comparison with other assays, this assay was performed at 30°C and the result obtained with the *dnaA*$^+$ pBR325 culture normalised to a value of 1.00. In each subsequent assay (such as comparing activities between different alleles) a control assay using a *dnaA*$^+$ pBR325 culture was included to allow comparative analysis. The results of this initial assay are shown in table 4.14. It is apparent that the presence of pND5 appears to have no effect on expression from the *dnaA* promoter in a *dnaA*$^+$ strain.

TABLE 4.14 Effect of pND5 on transcription from P*dnaA* in a *dnaA*$^+$ strain at 30°C.

STRAIN	PLASMID	Specific Activity (32 samples)	Doubling Time(mins)
TP91 *dnaA*$^+$	pND5	1.01 ± 0.106	45
	pBR325	1.00 ± 0.100	40

Specific activity (i.e. β-galactosidase activity per O.D.) shown, together with the standard deviation. Results normalised to TP91 pBR325 at 30°C = 1.00.

4.5.2 Transcription from P*dnaA* in a *dnaA46ts* strain

Expression from P*dnaA* was subsequently examined in a *dnaA46ts* strain. The strain used was TP88 (*lac* delete, *dnaA46ts*, λRB1 lysogen), which is isogenic with TP91. Transformants (pND5 or pBR325) were tested as above; in addition the temperature sensitive phenotype of TP88 pBR325 and the cold sensitive phenotype of TP88 pND5 were verified. Cultures were maintained as described above prior to sampling for β-galactosidase activity. TP88 pND5 was grown at 37°C until early log phase, then diluted back to 30°C or 42°C and growth followed for several hours while samples were taken. TP88 pBR325 was grown at 30°C until steady state growth was achieved, then diluted back to 30°C and 42°C and sampling begun immediately. The growth rates and normalised β-galactosidase figures are shown in table 4.15. Examining these figures, the slow down in growth due to the onset of cold sensitivity in TP88 pND5 at 30°C is apparent, although the expression from the *dnaA* promoter appears unchanged (this would tend to suggest that cold sensitivity may not be due to

increased binding of DnaAts protein to DNA). So too is the induction of β-galactosidase activity following thermal inactivation of the DnaA46ts protein in the non-suppressed strain (MM185 pBR325 at 42°C). In addition, compared to the *dnaA*$^+$ strain, increased expression from the *dnaA* promoter is seen in the *dnaA46ts* strain even at the permissive temperature, indicating that autoregulation is not fully functional at 30°C (a temperature permissive for growth nonetheless). Examining TP88 pND5, it is apparent that *groE* does not mediate suppression by causing over-expression of the mutant *dnaAts* gene, although a low level of increased expression is seen in MM185 pND5 at 42°C (far below that caused by thermal denaturation alone though). Indeed, expression is reduced to below the levels seen in a *dnaA*$^+$ strain at 30°C, suggesting perhaps that cold sensitivity may be due to a lack of DnaA protein. However, it should be remembered that a promoter assay system such as that employed here does not measure the actual amount of a particular protein, merely its presumed rate of synthesis. It is possible, for example, that reduced transcription from the *dnaA* promoter is due to an increased amount of DnaA protein, not as a result of an increased rate of synthesis, but because of a reduced rate of breakdown in a suppressed strain. However, it appears clear that *groE* does not suppress by increasing the expression of the mutant *dnaA46* gene.

TABLE 4.15 Effect of pND5 on transcription from P*dnaA* in a *dnaA46* strain[a]

	30°C	42°C
TP88 *dnaA46* pND5		
Specific Activity[b]	0.65 ± 0.08 → 0.67 ± 0.02	1.36 ± 0.21
Growth Rate[c]	38 → 120	40
TP88 *dnaA46* pBR325		
Specific Activity[b]	1.65 ± 0.23	1.32 ± 0.17 → 4.08 ± 0. 28
Growth Rate[c]	36	30 → 100

a. For TP88 pND5 (30°C) and TP88 pBR325 (42°C), two measurements for growth rate and promoter activity are shown. The first is immediatly following temperature shift, the second taken several hours later, when cold sensitivity (pND5) or cessation of growth (pBR325) had become apparent

b. Specific activity (i.e. β-galactosidase activity per O.D.) shown, together with the standard deviation. Results normalised to TP91 dnaA$^+$ pBR325 at 30°C = 1.00.

c. Doubling time of the culture in minutes.

4.5.3 Construction of isogenic *dnaAts* λRB1 lysogens

After the results obtained with the *dnaA*$^+$ and *dnaA46ts* alleles it was considered of interest to see if similar results were obtained with *dnaA167ts* (suppressed, but not cold sensitive) and *dnaA508* (not suppressed, not cold sensitive). If cold sensitivity is due to a reduction in *dnaA* expression, possibly this feature would be absent in the above alleles in conjunction with pND5. Unfortunately, *dnaAts* strains lysogenised with λRB1 and deleted for the *lac* operon were not available. However, it was considered that the isogenic *dnaAts* series MM181-MM190 might be suitable. These were available ready transformed with pND5 or pBR325. The problem with these strains is that they are all *lacZ*$^+$, such that expression of the chromosomal β-galactosidase could produce misleading results. However, on L-agar plates containing X-gal (but not the inducer IPTG) white colonies were seen, demonstating that under these growth conditions minimal expression was occurring. Lysogens of λRB1 were made by spotting the phage onto lawns of the bacterial strains, and then streaking from the centre of the resultant plaque onto L-agar containing X-gal. Lysogens were easily seen from their bright blue colour in contrast to the white appearance of non-lysogens.

4.5.4 Transcription from P*dnaA* in *dnaA167ts* and *dnaA508ts* strains

Experimental procedure for MM181 (*dnaA167ts*) and MM184 (*dnaA508ts*) λRB1 lysogens was essentially as described for TP88, except glucose at 0.2% was included in the media in order to further minimise the risk of expression of the chromosomal β-galactosidase gene. The growth rates and β-galactosidase activities are shown in table 4.16. Over-expression of *groE* in conjunction with *dnaA167* appears to make no difference to P*dnaA* expression, again indicating that increased expression of the *dnaAts* gene is not the mechanism by which suppression is occurring. Somewhat suprisingly, increased expression from P*dnaA* in conjunction with excess *groE* is seen with *dnaA508ts*, but since this allele is not suppressed this still agrees with the premise that suppression by *groE* is not due to increased production of the mutant DnaAts protein. Induction of expression from P*dnaA* is seen for both alleles following thermal inactivation of the mutant DnaAts protein. This effect is also present in the non-suppressed *dnaA508ts* pND5 strain (MM184) following a temperature shift.

TABLE 4.16 Effect of pND5 on transcription from P*dnaA* in a *dnaA167* and *dnaA508* strain.[a]

	30°C	42°C
MM181 *dnaA167* pND5		
Specific Activity[b]	0.98 ± 0.04	0.85 ± 0.19
Growth Rate[c]	62	48
MM181 *dnaA167* pBR325		
Specific Activity[b]	0.96 ± 0.06	1.02 ± 0.16⟶2.15 ± 0.22
Growth Rate[c]	60	40⟶90
MM184 *dnaA508* pND5		
Specific Activity[b]	1.38 ± 0.11	1.45 ± 0.27⟶2.12 ± 0.19
Growth Rate[c]	65	40⟶88
MM184 *dnaA508* pBR325		
Specific Activity[b]	0.58 ± 0.04	0.60 ± 0.09⟶1.03 ± 0.18
Growth Rate[c]	64	38⟶87

a. For strains MM181 pBR325, MM184 pBR325 and MM184 pND5 at 42°C, two measurements for growth rate and promoter activity are shown. The first immediately follows temperature shift, the second is taken c. 4 hours later.

b. Specific activity (i.e. β-galactosidase activity per OD) shown, together with the standard deviation. Results normalised to TP91 dnaA$^+$ pBR325 at 30°C = 1.00.

c. Doubling time of the culture in minutes.

4.5.5 Summary and Discussion

A summary of the expression from the *dnaA* promoter under the experimental conditions described in this section is presented in table 4.17. The results have been simplified as an aid to clarity. An increase in expression is seen with all *dnaAts* alleles following thermal inactivation of the mutant DnaAts protein; the presence of extra GroE appears to prevent this in the suppressed strains (*dnaA46ts, dnaA167ts*). The results at 30°C are somewhat amibigous. In a *dnaA*$^+$ strain, over-expression of *groE* appears to have no effect on expression from P*dnaA*; in a cold sensitive strain expression from P*dnaA* appears to be reduced. However, in a non-cold sensitive and

non-suppressed strain (*dnaA508ts*) an increase in expression is seen when pND5 is present. Thus *groE* suppresses both the autoregulatory and initiation functions of DnaA; the result with *dnaA508ts* demonstrates that loss of one function is accompanied by loss of the other (for this allele at least). This suggests that the mechanism by which DnaA acts is the same in both initiation and autorepression. From these results it appears clear that suppression by over-expression of *groE* is not due to an effect at the transcriptional level. The similar pattern of suppression seen between *groE* and *rpoB* is not due to increased expression of the mutant *dnaAts* gene, and may indicate that an interaction at the origin between RNA polymerase, GroE and DnaA is occurring.

TABLE 4.17 Summary of the effect of pND5 upon *dnaA* promoter activity

COMMENTS	ALLELE	30°C	42°C
	dnaA$^+$	1.0	N.T.
	dnaA$^+$ pND5	1.0	N.T.
SUPPRESSED, NOT	*dnaA167*	1.0	1.0→2.0
COLD SENSITIVE	*dnaA167* pND5	1.0	1.0
SUPPRESSED, IS	*dnaA46*	1.7	1.4→4
COLD SENSITIVE	*dnaA46* pND5	0.7	1.4
NOT SUPPRESSED,	*dnaA508*	0.6	0.6→1.0
NOT COLD SENSITIVE	*dnaA508* pND5	1.4	1.4→2.2

All data normalised to dnaA$^+$ = 1.0 at 30°C. N.T. = Not Tested.

However, several problems exist with this approach, which means that it can only be taken as a very approximate indication of the events occurring at a molecular level. Not the least of these being the fact that neither DnaA protein levels or indeed *dnaA* mRNA levels are being measured directly. Although GroE does not seem to suppress by increasing transcription of the *dnaA* gene, it is possible that it may increase the DnaAts protein levels by another mechanism. For example it could

stabilise the *dnaA* transcripts, improve translational efficiency of the message, or alternatively protect the DnaAts protein from degradation. All of these would have the effect of changing the DnaAts protein levels while being undetected by the P*dnaA-lacZ* fusion system in use above. Also, since *dnaA* is autoregulatory, changing either the overall level of DnaA protein in the cell (by one of the above mechanisms), or else changing its activity (possibly by causing it to bind more efficiently to DNA) would be expected to show the same result in the above system. i.e. A decrease in expression from the *dnaA* promoter. Therefore a way of visualising the DnaA protein directly would be more informative. This was one of the reasons which prompted the raising of antibodies against the DnaA protein (described in chapter 5). Regarding the possibility that GroE might either directly or indirectly protect the DnaAts protein from degradation, questions regarding the stability of the *dnaA-lacZ* translational fusion protein must be asked. Since the construct contains a small portion of the DnaA protein fused to a slightly truncated β-galactosidase (Braun *et al.* 1985), it is possible that this protein is itself partially unstable and subject to degradation. If so, then the figures for induction following thermal inactivation of the DnaAts protein may be an under-representation of the true figure. Finally, if GroE acted by protecting proteins in general from degradation, it is possible that the measured β-galactosidase activities are a mixture of transcription from P*dnaA* and effects on the stability of this fusion protein.

4.6 GroE and protein degradation

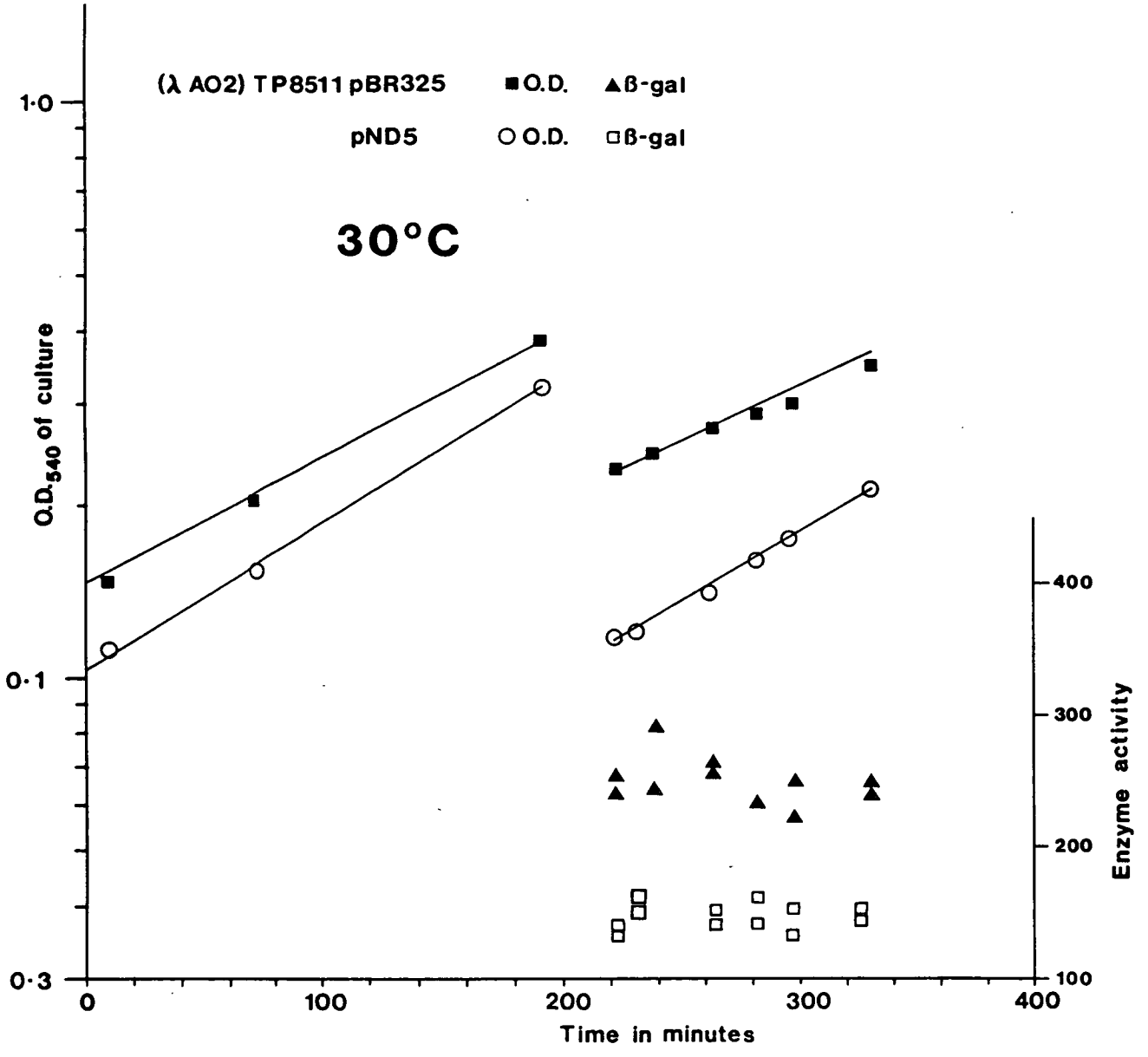
If, as suggested above, *groE* suppression of *dnaAts* mutants is mediated by protecting a partially denatured protein from degradation by cellular proteases, it is to be expected that other such proteins might be protected also. GroE might physically interact with the denatured protein thus protecting it from degradation. Alternatively, the large quantities of GroE present in suppressed strains may prevent induction of the heat shock response (removal of abberrant proteins being a function of this - for review see Neidhardt and VanBogelen 1987). i.e. Fool the cell into thinking it is already in a state of heat shock. Protection of proteins from degradation would be expected to be a fairly non-specific form of suppression; for example, how might allele specificity arise? A possible explanation would be that suppressible alleles are less severely denatured at high temperature than non-suppressible alleles. The mutant protein, although partially denatured, is still capable of participating in initiation, but is prevented from doing so as a result of degradation by proteases.

4.6.1 Stability of a β-galactosidase fusion protein in strains over-expressing *groE*

As an indication of whether over-expression of *groE* might act as a general inhibitor of proteases, it was decided to examine the stability of a β-galactosidase-*P.falciparum* fusion protein. This protein, encoded by the recombinant phage λAO2, exhibits a low level of β-galactosidase activity and is repotedly (Gordon Allan pers. comm.) unstable in *E.coli*. Strain TP8511 (*lac* delete) transformed with either pND5 or pBR325 (as a control) was lysogenised with this phage as previously described (section 4.5.3). The resultant colonies were very pale blue on X-gal plates when induced with IPTG, consistent with a low stability for the fusion protein. Since λAO2 is $c1^{857}$, lysogens were maintained at 30°C. Fresh overnights were diluted back the following day and growth maintained in early log phase until steady-state growth was achieved. Pulse induction of the fusion protein was achieved by pelleting the culture and resuspending in an equal volume of CAA medium containing IPTG (described in chapter 2). The culture was maintained at 30°C for 10 minutes prior to pelleting and washing twice with L-broth. The culture was resuspended in L-broth and growth continued as before at 30°C. Samples for β-galactosidase assays were taken at regular intervals. Growth curves and *b*-galactosidase activities are shown in figure 4.6. β-galactosidase activities are plotted as total rather than specific activities, since the breakdown of a fixed amount of protein is being monitored, which obviously should not increase as the optical density of the culture. The specific activity at the time of induction is shown also, to give an indication of the relative levels of induction (shown in Miller Units). Several points are apparent. (1) pND5 causes a noticable reduction in growth rate in this strain. (2) The fusion protein appears to be totally stable. (3) Level of β-galactosidase activity appears to be very low. Perhaps in previous observations (Gordon Allan pers. comm.) a low level of induction for this protein was mistaken for low stability.

Several problems exist with this approach which suggest that investigation of protein stability should be conducted directly rather than via enzyme assays. It is possible that changes to the β-galactosidase fusion protein might have positive as well as negative effects on its activity, although this was not apparent at the time of study. When the β-galactosidase-DnaA fusion protein described in chapter 5 was purified by affinity chromatography, the major purified protein was smaller than the intact fusion, suggesting that the intact fusion either did not possess β-galactosidase activity or that its activity was much less than that of smaller fragments. Therefore partial degradation of the intact protein might result in an increase in the

FIGURE 4.6 Effect of pND5 on the stability of a β-galactosidase-*P.falciparum* fusion protein



β-galactosidase total enzyme activity. See text for details.

β-galactosidase activity (section 5.4). Thus in this instance protection of the fusion protein would actually give totally the opposite result from that expected (i.e. less activity), and an incorrect interpretation of the data would result. As such, any investigation into the stability of aberrant or partially denatured proteins would

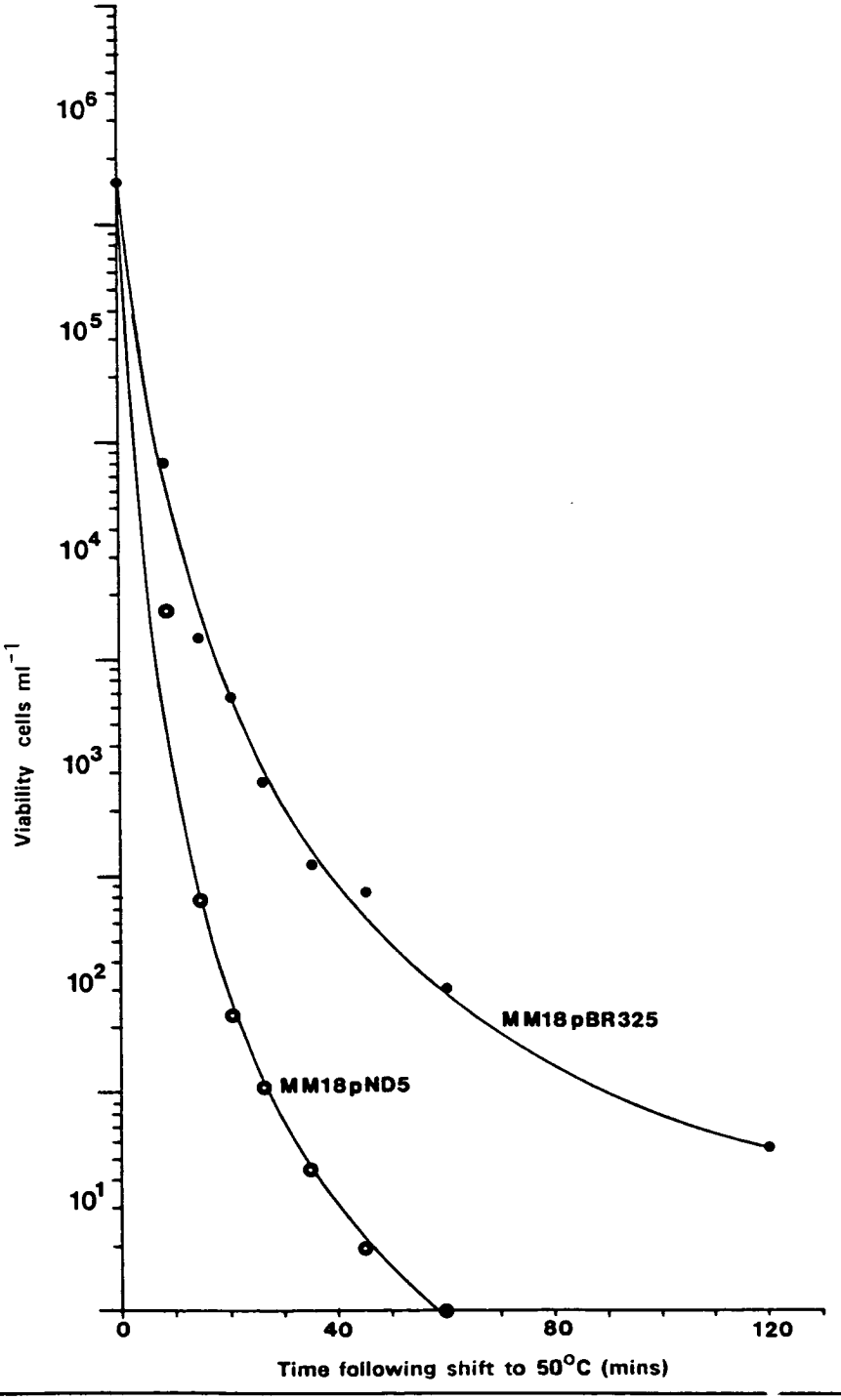
probably be best conducted by a direct visualisation of the protein in questin, by SDS-PAGE analysis at time intervals after induction for example.

4.6.2 Effect of heat-shock in cells over-expressing *groE*

It has been suggested that GroE might act directly to protect aberrant proteins from degradation by some kind of physical interaction. Another possibility is that over-expression of *groE* might prevent induction of the heat shock response, constituents of which are cellular proteases (Neidhardt and VanBogelen 1987). Thus the excess GroE might be acting in some kind of regulatory fashion. To test this it was decided to examine the heat shock response in cells over-expressing *groE* to see if any changes could be detected. Initially it was decided to examine the viability of cells containing pND5 or pBR325 (as a control) under heat-shock conditions, since a failure to induce the heat-shock response should be exhibited by a lower tolerance of high temperatures. Accordingly, strains MM18 pND5 and MM18 pBR325 were grown and maintained at 30°C until steady state growth was achieved. The cultures were then transferred to a 50°C water bath and samples taken at regular intervals for viable counts. The results are shown graphically in figure 4.7. Cells over-expressing *groE* show a reduced viability following heat-shock compared to the control strain, suggesting perhaps that the heat-shock response is not being properly induced. However, since *groE* is itself induced as part of the heat-shock response, it is possible that the reduced viability observed is due to induction of an already over-expressed protein perhaps placing too great a strain on the cells' metabolism.

As a further test of the heat-shock response in cells over-expressing *groE*, it was decided to examine the growth rate and cell size following heat-shock in MM18 pND5 and MM18 pBR325. A feature of the heat-shock response is the transient inhibition of cell division following a shift in temperature (Tsuchido *et al.* 1986). This would be expected to be absent if heat-shock were indeed inhibited in strains over-expressing *groE*. Accordingly, strains MM18 pND5 and MM18 pBR325 were grown and maintained at 30°C until steady state growth was achieved. The cultures were then diluted back and shifted to 46°C and growth was followed. In addition, immediately prior to and following heat shock, cell sizes were measured. These measurements are shown in figure 4.8. Prior to heat shock the growth rates of MM18 pND5 and MM18 pBR325 are essentially identical. Following transfer to 46°C the growth rates are markedly different though, that of MM18 pND5 being much reduced compared to MM18 pBR325, in agreement with the reduced viability for the pND5-

FIGURE 4.7 Effect of pND5 on host cell viability following heat shock



Data normalised to viability at time 0 = 1.6 x 10^6 cells ml^{-1} (the figure for MM18 pBR325). Value for MM18 pND5 at time 0 was 7 x 10^6 cells ml^{-1}.

containing cells following heat shock as was noted above. Is this the result of non-induction of the heat-shock response though? Examining the data on cell size this would appear not to be the case, since pND5-containing cells filament as per the control. Indeed, filamentation appears to occur over a more extensive time period, although this may be more a reflection of the much reduced growth rate rather than any specific effect on the duration of the response. It seems likely therefore, that induction of the heat shock response does indeed occur in cells over-expressing *groE*, although a more definitive answer to this question could probably be obtained if a detailed investigation into the proteins induced following heat shock (plus and minus pND5) were obtained. This would involve the radiolabelling of cellular proteins with S^{35}-methionine, followed by an examination of their levels using two dimensional gel electrophoresis. If protection of proteins does occur in a pND5-containing strain, it is more likely to be due to a specific interaction between GroE and the protein in question rather than by a more general suppression of the heat shock response. It would seem therefore, that investigations into the possibility that over-expression of *groE* might suppress *dnaAts* mutants by protecting the mutant DnaAts protein from degradation, will require direct visualisation of DnaA protein levels. Amongst other things, this was one of the reasons which lead to raising of antisera towards the DnaA protein, described in chapter 5.

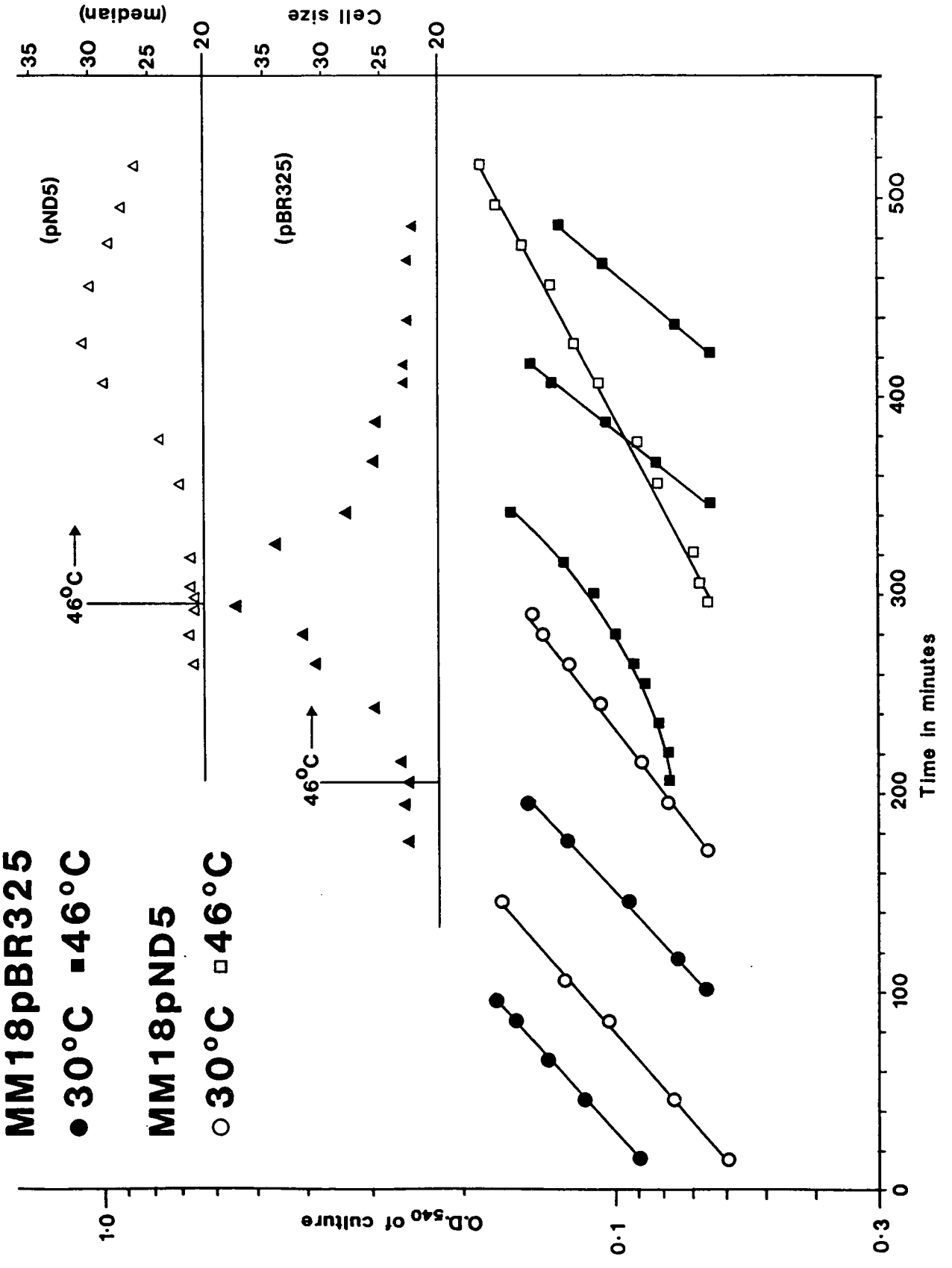
4.7 Summary and discussion of *groE* suppression

The experiments described in this chapter were performed with the objective of examining possible mechanisms of suppression in an attempt narrow down the most likely process by which suppression actually occurs. The main mechanisms by which suppression may be occurring are:-

- **Bypass**
- **Informational**
- **Increased expression of the mutant protein**
- **Protection of the mutant protein**:- (1) *Indirect* -prevents induction of the heat shock response. (2) *Direct* -interacts with mutant protein and protects it from proteases. (3) *Repairs heat-denatured protein*.
- **Interactive** (either a direct interaction with DnaA or via an intermediate protein)

The experiments conducted to examine each possibility and any conclusions which can be reached are now discussed.

FIGURE 4.8 Effect of pND5 on growth rate and cell size following heat shock



Bypass suppression: Since *dnaAam* mutations cannot be suppressed by over-expression of *groE* (togther with some *dnaAts* alleles) it would appear that suppression is not bypass in nature, and requires the presence of the intact DnaAts protein.

Informational Suppression: Evidence that *groE* may suppress at the translational level is provided by several lines of evidence. The *groE* gene products have been reported to be associated with ribososmes (Neidhardt *et al.* 1981), suggesting that an interaction may be occurring. Also, allele specificity should be seen with informational suppression, as is observed with *groE* suppression of *dnaAts* mutants (for example if all suppressed alleles contained the same mis-sense mutation). Additionally, strains containing alleles which, in combination with over-expression of *groE* result in cold sensitivity are also cold sensitive as merodiploids with $dnaA^+$. Since informational suppression should result in both mutant and non-mutant forms of the protein, this provides a convenient explanation of the cold sensitive phenotype seen with *groE* over-expression.

However, for a variety of reasons it would appear unlikely that informational suppression is taking place. Informational suppression is a fairly non-specific process, such that any temperature sensitive protein with the correct missense mutation should be suppressible. However, suppression by *groE* appears to be highly specific; although *dnaAts* and *ams* mutants are suppressible, over-expression of *groE* cannot suppress mutations in *dnaC1, dnaC2, dnaB252, dnaP18* together with a variety of nutritional markers (Jenkins *et al.* 1986). Work by Fayet *et al.* (1986) has additionally shown that temperature sensitive mutations *dnaB42, dnaB558, dnaC28, dnaG308, dnaK103, dnaK7* are not suppressed. If *groE* was acting in a fairly general manner, for example mimicking the effect of streptomycin (which results in the insertion of a variety of different amino acids; see Kurland 1984 for review), not only should a wide range of different mutations be suppressed, but in addition, the phenotypic side effects of such suppression should be severe (production of a wide range of mutant peptides from all translated messages, whether *dnaA* or not). If *groE* acted in a manner analogous to tRNA suppressors, inserting a different (i.e. functional) amino acid from that encoded by the mutant codon (Hill 1975), then a very narrow pattern of allele specificity should be seen, since the suppressor should only recognise two or three of the 61 possible missense codons. It is most unlikely that all the *dnaAts* mutant alleles which are suppressible by *groE* are of the same type. Rather, the structural clustering of the suppressible alleles suggests that location within the gene rather than the type of mutation *per se* determines whether it can be suppressed. Finally, although the

pattern of allele specificity seen for cold sensitivity suggests the production of mutant and non-mutant forms of the *dnaA* protein, it should be remembered that a similar pattern is also seen when transforming *dnaAts* strains with *oriC* plasmids. Thus, cold sensitivity can be caused by factors other than the formation of *dnaA*$^{+}$/*dnaAts* merodiploids.

Increased expression of the mutant protein: The possibility that suppression might be due to increased expression of the mutant *dnaAts* protein was suggested by two observations; (1) The pattern of allele specificity of *groE* suppression is exactly matched by some secondary mutations in the *rpoB* gene, suggesting that *groE* might act by causing RNA polymerase to transcribe *dnaA* at a higher level, and (2) over-production of mutant DnaAts protein has been reported to compensate for the *ts* phenotype and allow growth at 42°C (quoted in Atlung 1984). However, examination of transcription from the *dnaA* promoter showed that this was not the case in a suppressed strain. However, the possibility exists that over-expression of *groE* may increase the amount of DnaAts protein by some other means.

Protection of the mutant protein: Protection of the mutant DnaAts protein from degradation would have the same effect as increasing its rate of production, so it is possible that *groE* might be mediating suppression by this route. The possibility that over-expression of *groE* might be acting by preventing induction of the heat shock response was investigated; although cells were more sensitive to heat shock, they did appear to filament as per normal, suggesting that heat shock was being induced (although a more definitive investigation into this would require the use of protein gels and a measurement of the levels of induction of the various heat shock proteins). Another possibility is that GroE actually 'repairs' the defective protein, possibly by unwinding an incorrectly folded protein, allowing it to function and thus protecting it from proteases (this was discussed in the last chapter). Finally, it is possible that GroE might not actually alter the mutant protein, but rather physically protect it from proteases, presumably preventing them from gaining access to it. To investigate these latter two possibilities it was decided to obtain DnaA antiserum, since it was hoped that an accurate determination of the levels of DnaA protein, the presence of breakdown products, and the investigation of whether different forms of the DnaA protein existed between suppressed and non-suppressed strains would be possible.

Interactive suppression: Of all the possibilities, this appears the most likely, that of a direct interaction between GroE and DnaAts leading to suppression. Only this would satisfactorily explain the pattern of allele specificity seen, with the

locational clustering of the suppressed alleles suggesting a functional domain within the DnaA protein. The above possibilities would all be expected to be relatively pleiotropic in their effect, and should allow suppression of a wide range of otherwise unconnected mutations. The similar pattern of allele specificity seen with some *rpoB* mutants, the fact that both RNA polymerase and DnaA act at an early stage in the initiation process (possibly in complex), the previously discussed (section 1.6.3) suppression of an ssb-1 mutation by an allele of *groEL* (Ruben *et al.* 1988), plus the fact that *groE* mutants fail to replicate their DNA properly (Wada and Itikawa 1984), all suggest that some interaction between *oriC*, DnaA and RNA polymerase is occurring during initiation. Although it is not clear if cold sensitivity is due to a specific interaction at *oriC*, the clustering of effect and allele location within the gene again suggests some kind of structural interaction as being the cause. Finally, the fact that a truncated *groEL* protein would appear to be able to suppress more efficiently than the intact form would suggest an interaction between the two proteins, such that a secondary mutation in one is compensating for the original mutation in the other.

It was hoped that obtaining DnaA antibodies would help in examining the question of whether such a specific interaction existed. Would DnaA co-purify with GroE for example? Do GroE and DnaA interact at *oriC*, either *in vivo* or *in vitro*? Having covered the preliminary experiments designed to investigate the means of suppression of *dnaAts* mutations by over-expression of *groE*, it would appear that the most likely candidates are either some kind of interaction between GroE and DnaA, thus increasing the ability of the mutant DnaAts protein to function, or alternatively some kind of protection/repair of the mutant form at high temperatures, thus increasing the effective amount of protein. It is possible that both hypotheses are partially correct. Chapter 5 now describes the cloning of *dnaA*, the construction of a β-galactosidase-DnaA fusion protein, and the raising of DnaA antiserum.

CHAPTER 5

Cloning of *dnaA*, the construction of a B-galactosidase-DnaA fusion protein, and the raising of DnaA antisera

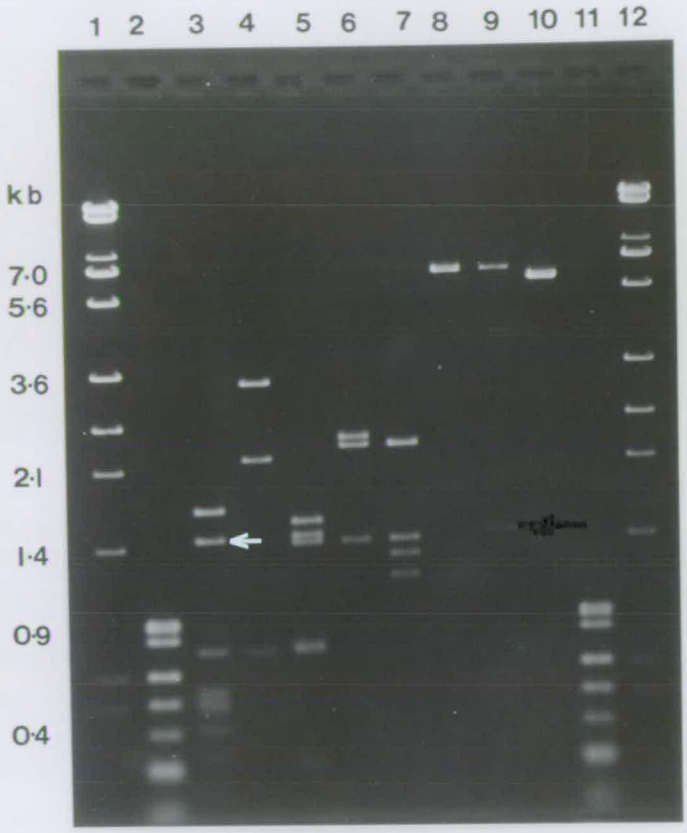
5.1 Cloning of *dnaA*

In order to raise antibodies against a particular protein, it is first desirable to obtain as pure a preparation of that protein as possible. In the case of an *E.coli* protein, this is greatly facilitated by the use of a strain which over-produces the protein in question. To achieve this the gene coding for the protein to be purified can be cloned downstream of a powerful and inducible promoter, cloned into either a multicopy vector or a λ phage cloning vehicle (for practical aspects of this see Glover 1984). Obviously to accomplish this first requires that the gene coding for the desired protein be cloned and available for such genetic manpulations; i.e. at least partially characterised with regard to the position of restriction endonuclease sites and the direction of transcription of the gene. The *dnaA* gene has been both cloned and sequenced, and has been shown to reside on a 3.9kb *Hin*dIII fragment (Hansen *et al.* 1982). Since an *E.coli Hind* III library cloned into pBR325 was available in this laboratory, it was considered a relatively straightforward matter to transform the library into a *dnaAts* strain at a temperature restrictive for growth and screen the resulting transformants for the presence of a *dnaA* containing plasmid. Although it was anticipated that such an approach might result in the isolation of extrachromosomal suppressors of *dnaA* (such as resulted in the isolation of *groE* for example), the knowledge that *dnaA* resides on a 3.9kb *Hin*dIII fragment was expected to render identification of the *dnaA* gene relatively straightforward.

5.1.1 Isolation of pJM87-01

Initially it was decided to transform the *E.coli Hind*III chromosomal library into MM184 and select for temperature-resistant transformants. It was decided to use this strain since it carries the *dnaA508ts* allele, which is irreversibly denatured upon thermal inactivation -in contrast to the majority of *dnaAts* alleles (Hansen *et al.* 1984)- and therefore seemed less likely to result in the isolation of repressors or revertants (assuming that the irreversible nature of the allele suggests a more severe mutation). The strain was transformed with a *Hin*dIII library cloned into pBR325, and transformants were selected on ampicillin plates at both 30°C and 42°C. While 2 transformants appeared at the higher temperature, approximately 2-3000 colonies were isolated on the plate incubated at 30°C. This demonstrated the integrity of the library, and additionally gave the expected number of complementing plasmids out of the entire complement of recombinants in the library. (Since the *E.coli* chromosome is approximately 4000kb in length approximately 1 in 1000 plasmids should contain the

FIGURE 5.1 Restriction analysis of pJM87 and pJM87-01



The HinfI dnaA fragment used to make pJM88 is shown marked with an arrowhead.

Track 1: λcI^{857} AccI size standards
Track 2: pBR325 AluI size standards
Track 3: pJM87 HinfI
Track 4: pJM87 HincII
Track 5: pJM87 HincII-PvuII
Track 6: pJM87 PvuII
Track 7: pJM87 HindIII-PvuII
Track 8: pJM87 HindIII
Track 9: pJM87-01 HindIII
Track 10: pBR325 HindIII
Track 11: pBR325 AluI size standards
Track 12: λcI^{857} AccI size standards

correct fragment). The two 42°C transformants were purified (streaked to single colonies) and grown up in liquid media prior to screening of the plasmids. Gel electrphoresis showed them to contain a 1.5kb *Hin*dIII insert in addition to the vector band (figure 5.1). Since this was evidently not the 3.9 kb *dnaA* fragment expected, this suggested the isolation of an extrachromosomal suppressor. As both plasmids were apparently identical, this suggested that suppression was probably due to the plasmid insert fragment rather than the simultaneous isolation of a chromosomal suppressor. As a check of this, both plasmids were back-transformed into MM184, selecting for transformants at both 30°C and 42°C. As many transformants were isolated at 42°C as at 30°C, indicating that suppression was indeed plasmid mediated, and on the face of it, highly efficient. This plasmid was called pJM87-01, and comparison of the insert fragment (1.5kb) with other high-copy suppressors of *dnaA* (Takeda and Hirota 1982, Projan and Wechsler 1981), suggested the isolation of a new extrachromosomal suppressor of *dnaA*. This plasmid is discussed further in section 5.1.4.

5.1.2 Isolation of partial suppressors of *dnaA*

After the isolation of pJM87-01, it was decided to further screen the *Hin*dIII chromosomal library for the presence of the correct insert. Since only two temperature resistant colonies had been originally isolated, it was felt likely that the correct insert had been missed by chance rather than that it was not represented in the library. It was, however, considered possible that the irreversible denaturation of the DnaA508ts protein might prejudice against complementation by a second copy of the *dnaA* gene. Therefore mutant alleles *dnaA46ts* (MM19), *dnaA167ts* (MM181), *dnaA5ts* (MM182) and *dnaA204ts* (MM183) were transformed with the library, and plated at 30°C and 40°C as before. Apart from the MM19 42°C transformants (9), about 20 transformants of each strain were isolated at 40°C, compared to c.5-10,000 at 30°C. Nine MM19 and 16 of the other transformants were streaked to purity at 42°C, the results of this being shown in table 5.1. Colonies which grew convincingly upon restreaking were were subsequently grown in liquid culture (at 40°C) and plasmid DNA preparations made. Analysis of the recombinant plasmids revealed the 6kb pBR325 vector fragment together with a variety of different sized inserts, ranging from 1.2-18kb. Some of the inserts appeared several times, both in the same strain and isolated in a different background. The recombinant plasmids were grouped together into similar size catagories and the inserts electrophoresed on the same agarose gel to differentiate similar-sized from apparently identical inserts. This allowed classification of the *Hin*dIII insert fragments into 19 size classes (shown in table 5.2).

TABLE 5.1 Isolation of partial suppressors of *dnaAts*

STRAIN	MUTATION	ORIGINAL NO. OF TEMP^R TRANSFORMANTS	COLONIES ABLE TO GROW AFTER RESTREAKING
MM19	*dnaA46*	9	1
MM181	*dnaA167*	16	14
MM182	*dnaA5*	16	13
MM183	*dnaA204*	16	10

Colonies grown on LBamp plates at 40°C.

Inserts which appeared only once were considered less likely to contain *dnaA* or be *dnaA* suppressors, and more likely to represent host cell reversion/suppression. It can be seen from table 5.2 that *Hin*dIII inserts of the correct size for *dnaA* (classes 5,6 and 7) are only represented once in these isolates, suggesting perhaps that successful cloning of this gene had not been achieved. However, insert classes 8,9,11,12 and 18 appear several times, either as independent isolates within the same strain, or indeed isolated in different *dnaAts* hosts. For example, the insert of 4.7kb (class 9) is seen in MM181, MM182 and MM183. Such multiple isolates suggests the presence of real extrachromosomal suppression/complementation rather than simple host cell reversion. Unfortunately however, although it appeared likely that a plethora of potential suppressors had been isolated, successful cloning of the *dnaA* gene did not appear so likely.

This was further confirmed when these plasmids were back-transformed into the host strains in which they had originally been isolated. Transformants were selected at both 30°C and 40°C, and the number of colonies obtained at each temperature counted. These results are shown in table 5.3. Several transformants of each type were then streaked at both 30°C and 40°C. The extent of growth at this temperature is measured on a points rating; +++ being equivalent to growth of the *dnaA*$^+$ parental at this temperature (MM18pBR325), and - being equivalent to the growth of the *ts* strain containing pBR325. All 40°C transformants regrew when streaked at this temperature and are not shown in the table. It was not possible to obtain transformants with some of the plasmids at either temperature, and these are not considered further. Most of the plasmids appear to be 'partial'

TABLE 5.2 Insert classes of high copy *dnaAts* suppressors

CLASS OF INSERT SIZE OF INSERT (kb)		1 1.15	2 3.2	3 3.3	4 3.5	5 3.8	6 4.0	7 4.1	8 4.4	9 4.7	10 5.1	11 5.2	12 6.2	13 8.1	14 8.4	15 8.6	16 9.4	17 10.0	18 13.5	19 18.0
NUMBER OF	MM19				1															
APPEARANCES	MM181		1		1			1	3	2		2			1	1		1		1
IN EACH	MM182	1				1	1		3	1	3		1	1					1	
STRAIN	MM183			1						1		3	3				1		1	
	TOTAL	1	1	1	2	1	1	1	6	4	3	5	4	1	1	1	1	1	2	1

TABLE 5.3 Back-transformation of suppressing plasmids

INSERT CLASS	STRAIN IN WHICH ISOLATED	NO. OF COLONIES OBTAINED FOLLOWING BACK TRANSFORMATION 30°C	40°C	GROWTH OF 30°C TRANSFORMANTS AT 40°C[a]
1	MM182	500	-	-
2	MM181	80	30	++
3	MM183	41	-	++
4	MM19	38	-	++
	MM181	200	40	++
5	MM182	21	-	-
6	MM182	20	-	-
7	MM181	85	3	++
8	MM181	22	1	-
	MM182	300	1	++
9	MM181	500	30	+
	MM182	-	-	NT
	MM183	80	1	+++
10	MM182	-	-	NT
11	MM181	200	44	++
	MM183	2	-	+++
12	MM182	200	-	++
	MM183	61	2	++
13	MM182	200	1	+

Table 5.3: (continued)

INSERT CLASS	STRAIN IN WHICH ISOLATED	NO. OF COLONIES OBTAINED FOLLOWING BACK TRANSFORMATION 30°C	40°C	GROWTH OF 30°C TRANSFORMANTS AT 40°C[a]
14	MM181	50	50	+++
15	MM181	40	23	+++
16	MM183	-	-	NT
17	MM181	31	-	++
18	MM182	55	-	++
	MM183	1	-	++
19	MM181	37	41	++

a. Subjective estimate of growth (dnaA$^+$ control = +++) NT = Not tested.

high-copy suppressors of *dnaAts*, able to allow reasonable growth at a normally restrictive temperature. However, it seems that the efficiency of this suppression is very poor if selection for suppression directly following transformation is attempted. This is similar to the findings of Projan and Wechsler (1981), who isolated several high-copy *dnaAts* suppressors which were only able to grow at the restrictive temperature if pre-grown at 30°C following transformation. Some suppressors appear more efficient than others, in particular classes 14, 15 and 19, where the transformation frequency at 40°C is similar to that obtained at 30°C. Unfortunately however, the insert classes of the correct size to contain *dnaA*, classes 5 and 6, were unable to complement/suppress *dnaAts* and it was therefore concluded that successful isolation of *dnaA* had not been achieved. Further characterisation of these plasmids was not performed, and they were named according to the clone number and strain in which they had originally been isolated (e.g. pJM182-1; clone number 1, originally isolated in MM182).

5.1.3 The cloning of the *rpoB* genes in high copy number: ability to act as a partial suppressor of *dnaAts*

During the course of this work it was discovered that *rpoBC* appears able to partially suppress *dnaAts* mutants when cloned in high copy number, similar to the situation observed with the above plasmids. Plasmid pHR3, which contains the *rpoBC* genes cloned into pBR322 had been transformed into the low copy strain JM21 for use in a complementation experiment (section 7.3). As a control, the plasmid was also transformed into the isogenic parental MM185. Both strains are *dnaA46ts*, while JM21 contains the *pcnB18* mutation. Prior to growth of the strains for copy number determinations (see section 7.2.5), both strains, together with JM21 pBR325 and MM185 pBR325 were streaked out on L-Agar + amp50 plates. By mistake the cultures were incubated at 37°C instead of 30°C. However, upon examination the next day it was noted that the strains containing pHR3 were able to grow significantly better than the pBR325 controls. This was apparent for both MM185pHR3 and JM21pHR3. Both strains were subsequently restreaked at 30°C, 37°C, 40°C and 42°C, and growth compared. The results of this suggested that growth at higher temperatures was better in the pHR3-containing culture. As a more definitive test of this, it was decided to transform pHR3 into the isogenic *dnaAts* series MM181-MM190, and compare growth at the different temperatures. The strains were transformed at 30°C, and then streaked out at 30°C, 37°C, and 40°C and 42°C, together with pBR325 containing cultures as a control, and growth compared. This is shown in table 5.4. Strains containing pHR3 are clearly able to grow better than the pBR325 controls, and this affect is seen for all the alleles tested. Thus the effect is clearly different from that seen with suppression of *dnaAts* by *mutations* in *rpoB*, which demonstrates allele specificity (Atlung 1984, Schaus *et al.* 1981b). I have termed the effect 'partial' suppression, since the presence of pHR3 does not appear to increase the upper temperature at which the *dnaAts* strain is able to grow (none of the pHR3 containing cultures was able to grow at 42°C for example). However, at the partially restrictive temperature of 37°C, pHR3-containing colonies are for the most part, significantly larger than the controls.

With regard to the plasmids described in section 5.1.2, and their 'partial' suppression phenotype, it was considered possible that pJM181-14 might contain the 10.1kb *Hin*dIII *rpoBC* fragment present in pHR3. Both fragments are of a similar size, and since a detailed restriction map of pHR3 was available (Richard Hayward pers comm.), the plasmids were digested with a variety of restriction enzymes and the resulting restriction pattern compared. However, despite the similarity in insert size,

TABLE 5.4 Partial suppression of *dnaAts* mutations by pHR3

STRAIN	PLASMID	30°C	37°C	40°C
VIABILITY AND COLONY DIAMETER (mm)				
MM181 *dnaA167*	pHR3	++ 1.0	++ 1.5	+/- 0.1-1.0
	pBR325	++ 1.0	++ 1.5	-
MM182 *dnaA5*	pHR3	++ 1.0	++ 1.2	+/- 0.1-0.3
	pBR325	++ 1.0	+ 0.1	-
MM183 *dnaA204*	pHR3	++ 1.0	++ 1.5	+/- 0.1-1.0
	pBR325	++ 1.0	++ 1.5	-
MM184 *dnaA508*	pHR3	++ 1.0	++ 1.5	+/- 0.1-1.0
	pBR325	++ 1.0	++ 1.5	-
MM185 *dnaA46*	pHR3	++ 1.0	++ 1.5	+/- 0.1-0.3
	pBR325	++ 1.0	+/- 0.1	-
MM186 *dnaA203*	pHR3	++ 1.0	++ 1.5	+/- 0.1-1.0
	pBR325	++ 1.0	++ 1.5	-
MM187 *dnaA602*	pHR3	++ 1.0	++ 1.2	+/- 0.1-0.3
	pBR325	++ 1.0	+/- 0.1	-
MM188 *dnaA601*	pHR3	++ 1.0	++ 1.2	+/- 0.1-0.3
	pBR325	++ 1.0	+/- 0.1	-
MM189 *dnaA604*	pHR3	++ 1.0	++ 1.2	+/- 0.1-1.0
	pBR325	++ 1.0	+ 0.2	-
MM190 *dnaA606*	pHR3	++ 1.0	++ 1.2	+/- 0.1-1.0
	pBR325	++ 1.0	++ 1.2	-

the restriction patterns of the two plasmids when digested with a variety of enzymes were markedly different, indicating that pJM181-14 did not in fact contain the 10.1kb *Hin*dIII *rpoBC* fragment of pHR3 (data not shown).

How might pHR3 suppress *dnaAts* mutations? The 10.1kb *Hin*dIII insert in pHR3 contains the genes *rpoB, rpoC, rplJ* and *rplK*, and these encode respectively the β and β' subunits of RNA polymerase, and the ribosomal proteins L10 and L11 (Newman and Hayward 1980). Secondary mutations in *rpoB* are able to suppress *dnaAts* mutations, although this suppression is allele specific in comparison to that observed above; the mechanism is presumably different. If suppression by pHR3 is due to over-expression of *rpoB* this could be mediated via a variety of routes (e.g. increased transcription of the mutant *dnaAts* allele, interactions at the origin of replication). Alternatively, increased expression of *rpoC* may be responsible for this phenotype, although this appears less likely perhaps since suppression of *dnaAts* by secondary mutations in this gene has not been reported. It appears unlikely that over-expression of one or more of the ribosomal structural proteins is responsible for this phenotype. Interestingly enough, the cloning of the *rpoBC* genes in high copy number only leads to a modest increase in the intracellular levels of their gene products, incommensurate with the increased gene dosage (Bedwell and Nomura 1986, Meek and Hayward 1986). This would appear to be due to the presence of a feedback loop which prevents the accumulation of excessive amounts of these RNA polymerase subunits. In view of the partial suppression phenotype of pHR3 suppression, one might speculate on whether more efficient expression of these genes might perhaps lead to a more complete suppression of *dnaAts* mutations.

Taking these results concerning pHR3, together with those obtained with the suppressor plasmids described in section 5.1.2 would tend to suggest that a large number of potential extrachromosomal suppressors of *dnaAts* exist, possibly indicating the involvement of the *dnaA* gene product with a large number of different proteins in a variety of cellular processes.

5.1.4 Characterisation of pJM87-01, and construction of a deletion derivative pJM87

Following on from the above work describing 'partial' suppressors of *dnaAts*, it was decided to further characterise pJM87-01, since this appeared to function highly efficiently as an extrachromosomal suppressor of *dnaA508ts*. The plasmid was transformed into the isogenic *dnaAts* series MM181-MM190, with plamid pBR325 included as a control. Transformants were selected at both 30°C and 40°C, and the figures shown in table 5.5 (10^0 dilution) represent the number of transformants obtained with 50ng of plasmid DNA. As expected, no pBR325 transformants were obtained at 40°C, and this is omitted from the table for the sake of clarity.

TABLE 5.5 Transformation of pJM87-01 into isogenic *dnaAts* series MM181-190

STRAIN	PLASMID	TEMP	NO.TRANSFORMANTS	COLONY DIAMETER
MM181 *dnaA167*	pJM87-01	40°C	≈2000	1.2
	pJM87-01	30°C	≈2000	0.5
	pBR325	30°C	≈200	0.5
MM182 *dnaA5*	pJM87-01	40°C	≈2000	1.2
	pJM87-01	30°C	≈2000	0.5
	pBR325	30°C	≈150	0.5
MM183 *dnaA204*	pJM87-01	40°C	≈3000	1.2
	pJM87-01	30°C	≈3000	0.5
	pBR325	30°C	≈300	0.5
MM184 *dnaA508*	pJM87-01	40°C	≈2000	1.2
	pJM87-01	30°C	≈2000	0.5
	pBR325	30°C	≈200	0.5
MM185 *dnaA46*	pJM87-01	40°C	≈2000	1.2
	pJM87-01	30°C	≈2000	0.5
	pBR325	30°C	≈200	0.5
MM186 *dnaA203*	pJM87-01	40°C	≈2000	1.2
	pJM87-01	30°C	≈2000	0.3
	pBR325	30°C	≈200	0.5
MM187 *dnaA602*	pJM87-01	40°C	≈100	1.2
	pJM87-01	30°C	≈500	0.1
	pBR325	30°C	11	0.5

Table 5.5: (continued)

STRAIN	PLASMID	TEMP	NO.TRANSFORMANTS	COLONY DIAMETER
MM188 *dnaA601*	pJM87-01	40°C	≈200	1.2
	pJM87-01	30°C	≈500	0.1
	pBR325	30°C	40	0.5
MM189 *dnaA604*	pJM87-01	40°C	≈200	1.2
	pJM87-01	30°C	≈400	0.1
	pBR325	30°C	21	0.5
MM190 *dnaA606*	pJM87-01	40°C	≈300	1.2
	pJM87-01	30°C	≈700	0.1
	pBR325	30°C	33	0.3

Transformants selected on LBamp50 plates ater overnight (c.18hrs) growth at the appropriate temperature.

Two points are immediately apparent upon examination of the data shown in table 5.5. Firstly, pJM87-01 appears to be an exceptionally efficient extrachromosomal suppressor of *dnaAts*, both in the fact that as many transformants were isolated at 40°C as at 30°C, and also since all the alleles tested appear to be suppressible. Secondly, the transformation frequency obtained with this plasmid appears very high compared to the pBR325 control. It is also interesting to note that pJM87-01 appears to cause cold sensitivity in conjunction with certain of the *dnaAts* alleles (the pattern of allele specificity being similar to, while not identical with that displayed by over-expression of *groE* or the presence of the *oriC* plasmid pHP6; section 4.4).

Since pJM87-01 appeared to be able to efficiently suppress all the *dnaAts* alleles tested, the next obvious question was to test whether it could suppress a *dnaAam* mutant. This was examined by transforming the plasmid into strain NS388, which is *dnaAam366* and contains a temperature sensitive tRNA suppressor (Schaus *et al.* 1981a). Transformants were isolated at both 40°C and 30°C, and the figures shown in table 5.6 (10^0 dilution) represent the number of transformants obtained per 50ng of

plasmid DNA. Again, a high transformation frequency compared to the pBR325 control is seen, together with the ability of pJM87-01 to suppress a *dnaAam* mutation.

TABLE 5.6 Transformation of pJM87-01 into a *dnaAam* strain

STRAIN	PLASMID	30°C	40°C
NO. OF TRANSFORMANTS			
NS388	pBR328	220	-
dnaAam366	pJM87-01	≈3000	≈3000

Selected on LBamp50 plates.

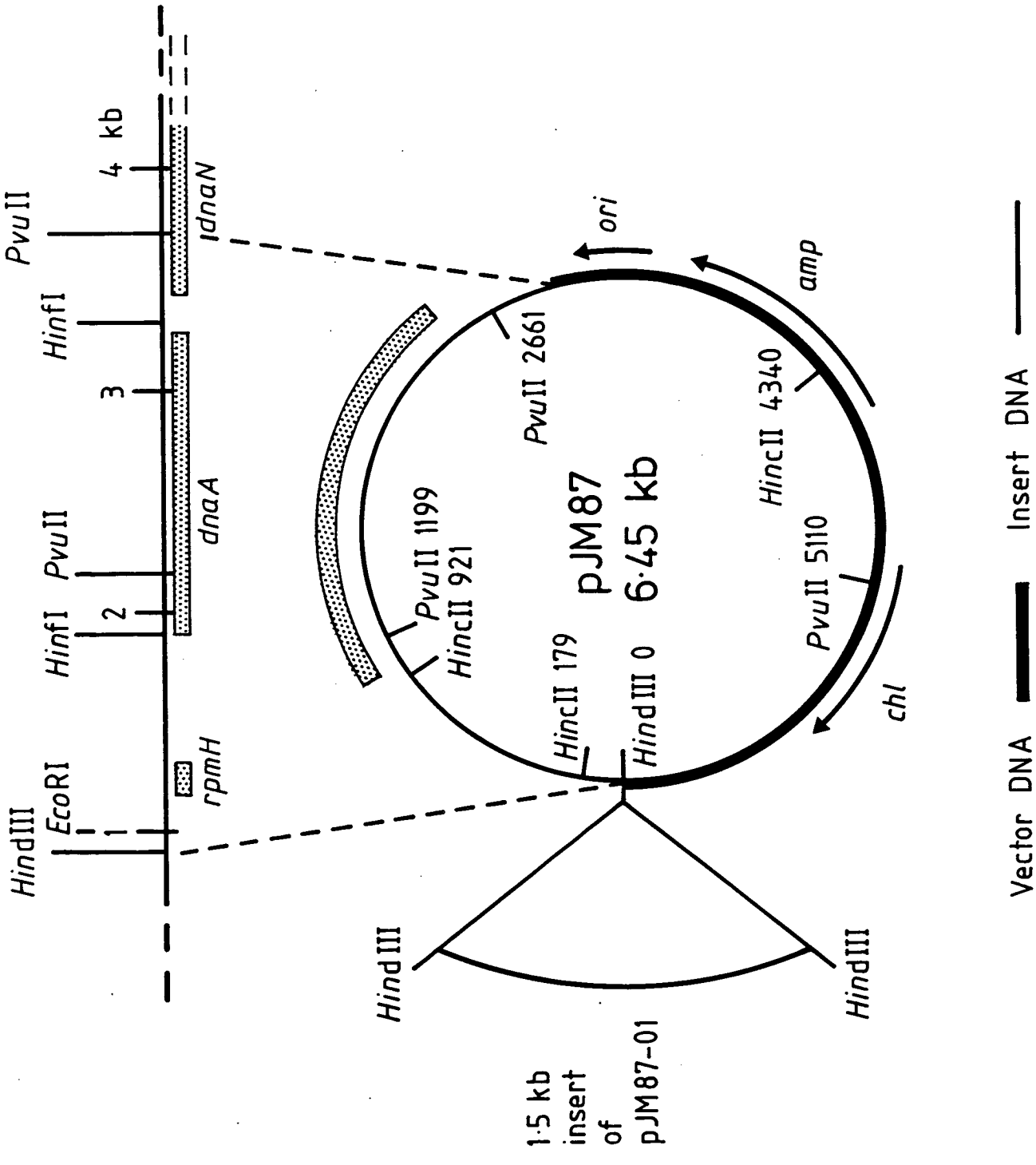
These results were suprising, since they suggested that pJM87-01 was acting as a bypass suppressor of *dnaA*. Bypass suppressors of *dnaA* have been reported before, but these have been exclusively chromosomal in location, and involve replication from an origin other than *oriC*, either by replication from an integrated DnaA-independent plasmid (integrative suppression), or via RNAaseH independent secondary origins in a *sdrA* mutant (Kogoma and von Meyenburg 1983, Molin and Nordstrom 1980). Replication from *oriC* is totally DnaA dependent (Hirota *et al.* 1970, Fuller *et al.* 1981), so for pJM87-01 suppression to be bypass in nature would suggest that over-expression of some protein product from this plasmid is presumably causing replication to occur from a different replication origin. The most logical explanation appeared to be that pJM87-01 contained the *dnaA* gene and that complementation rather than suppression was taking place. However, *dnaA* is contained within a 3.9kb *Hin*dIII fragment, and the insert fragment in pJM87-01 (1.5kb; figure 5.1) is too small to encode the *dnaA* gene even if a deletion removing all of the non-*dnaA* DNA had somehow occurred during cloning.

However, some clues regarding the ability of pJM87-01 to suppress *dnaAts* came when it was noticed that the *Hin*dIII vector fragment of this plasmid appeared very slightly larger than *Hin*dIII linearized pBR325, the parental of this plasmid (see figure 5.1). This suggested that additional DNA besides the 1.5kb *Hin*dIII insert fragment was contained within this plasmid. Perhaps this might be of importance

regarding the ability of pJM87-01 to suppress *dnaAts* mutations? The antibiotic resistances of pJM87-01 were examined, and it was found to confer ampicillin and chloramphenicol resistance, but not tetracycline resistance. Since the *Hin*dIII site in pBR325 is not within the tetracycline structural gene, this suggested the removal of pBR325 DNA and its replacement with some new sequence rather than the simple aquisition of a hundred or so bases of additional DNA. To further examine this, it was decided to reclone the 1.5kb insert into a new plasmid vector and see if it was still able to suppress *dnaA* mutations in the absence of this altered vector fragment. Additionally, the vector fragment deleted of this insert was re-ligated to discover if it was able to suppress on its own.

In the initial cloning experiment it was attempted to reclone the 1.5kb insert fragment into the *Hin*dIII site of pBR325; this construct was not obtained. However, it was regarded superflous to repeat this experiment, since plasmid pJM87 (pJM87-01 deleted of the 1.5kb *Hin*dIII fragment) was obtained, and shown to be capable of suppressing *dnaAts* mutations on its own (data not shown). How much pBR325 DNA had been replaced in pJM87, and what had it been replaced with? Restriction mapping of the plasmid was carried out (figure 5.1 and data not shown), and since the DNA sequence of the *dnaA* gene is known, it was possible to look for specific DNA fragments. This showed that pJM87 did in fact contain the *dnaA* gene, with about 2kb of pBR325 DNA being replaced, resulting in a plasmid of a total size of 6.45kb (in contrast to the size of pBR325, 6kb). The entire tetracycline resistance gene is deleted in this plasmid, together with the coding sequence for the Rop/Rom protein, which acts to negatively regulate plasmid copy number (Twigg and Sherrat 1980). This explained the high transformation frequencies previously observed with this plasmid in comparison to the Rom/Rop$^+$ parental pBR325. Additionally, this partially explained the pattern of allele specificity seen with the cold sensitivity caused by pJM87-01 in conjunction with certain *dnaAts* alleles, since many *dnaAts*/*dnaA*$^+$ merodiploids are known to be cold sensitive (Hansen *et al.* 1984). However, although similar, the pattern of alleles rendered cold sensitive by pJM87-01 is slightly different to that observed by Hansen *et al.*, possibly reflecting the presence of the *dnaA*$^+$ gene on a high copy vector in the former instance rather than in a 1:1 ratio with the mutant allele as was observed by these authors (the *dnaA*$^+$ gene being contained on a *l* phage). Further confirmation that this plasmid did indeed contain the *dnaA* gene was obtained by probing to the 1.5kb *Hin*fI *dnaA* fragment subsequently used to make the *dnaA-lacZ* gene fusion (section 5.2.2) with a *dnaA* fragment from the plasmid pBC32 (Churchward *et al.* 1983; data not shown). A restriction map showing the extent of DNA from the *dnaAN* region cloned into pJM87 is shown in figure 5.2.

FIGURE 5.2 Restriction map of pJM87



It is unclear how plasmid pJM87-01 arose. Presumably a double insert into the *Hin*dIII site of pBR325 occurred followed by a deletion of DNA from the the recombinant plasmid. Since the *dnaA* gene does not have a 1.5kb *Hin*dIII fragment immediately upstream of the site shown at 0kb in pJM87 (Hansen and von Meyenburg 1987), this would suggest the cloning of a totally unconnected DNA fragment in pBR325 alongside the 3.9kb *Hin*dIII *dnaA* fragment. Additionally, the fact that the 3.9kb fragment was not recovered intact from the chromosomal library would tend to suggest that the presence of this fragment in a high copy vector is somehow selected against. However, characterisation of pJM87 fulfilled the objectives of this part of the project, if in a rather circuitous way; that of the cloning of the *dnaA* gene prior to further work regarding the purification of its protein product.

5.2 Construction of a *dnaA-lacZ* gene fusion in a high-copy vector

5.2.1 Introduction

It was hoped that raising of anti-DnaA sera would help to answer two specific questions regarding *groE* suppression of *dnaAts* mutants. Firstly, to what extent DnaA protein levels are affected in a GroE over-producing strain, and secondly, to examine the stability profile of the DnaA protein under conditions of such over-expression, for both the wild-type and temperature sensitive versions. Additionally, it was hoped that more fundamental questions concerning the role of DnaA in the cell might be addressed.

It was anticipated that investigation of such questions would probably involve the use of the technique of Western Blotting; the electrophoresis of protein extracts on SDS-polyacrylamide gels followed by electrophoretic transfer onto nitrocellulose filters (for review see Towbin and Gordon 1984, Daneels *et al.* 1986). These could then be incubated with the appropriate antibodies and bands corresponding to the DnaA protein or its breakdown products visualised. This should hopefully allow investigation into both the level and stability of the DnaA protein. It was this anticipated application of the anti-DnaA sera which primarily determined the method of purification chosen.

Antibodies can be either polyclonal or monoclonal in nature. The advantages of monoclonal antibodies include their unlimited supply and defined specificity (for applications see Mole and Lane 1987); however, the cost and the inherant complexity of their production argued against their being used in this application. Although monoclonal antibodies recognise and interact with a specific unique epitope, such

specificity does not eliminate the possiblility of cross-reactivity and consequently aberrant results during their use (Kohler and Milstein 1975, Lerner 1982). Finally, and perhaps the most crucial factor in determining their suitability for such work, the use of anti-DnaA antibodies was specifically required to visualise DnaA breakdown products in the cell. Monoclonal antibodies, by their very definition, would be unsuitable for visualising a varity of DnaA protein fragments, possibly with no common epitopes.

Therefore it was decided to raise polyclonal antisera against purified DnaA protein. Again, two alternatives presented themselves. Whether to use purified intact native DnaA protein as an immunogen, or to prepare a β-galactosidase-DnaA fusion protein. Purification of native DnaA protein has been reported previously (Chakraborty *et al.* 1982, Fuller and Kornberg 1983), and an improved purification procedure has recently been developed (Sekimizu *et al.* 1988). These procedures result in a DnaA preparation which is greater than 90% pure as judged by SDS-polyacrylamide gel electrophoresis, and produce a physiologically active protein. However, as has been discussed above, it was anticipated that DnaA antisera would primarily be used to visualise SDS-denatured DnaA protein in a Western Blotting procedure, and therefore use of physiologically active DnaA protein to raise antisera was considered unnecessary. Indeed, it was considered likely that use of an SDS-denatured antigen would probably be advantageous under these conditions.

Since physiologically active DnaA was not required, it was decided to purify the DnaA protein as part of a β-galactosidase fusion protein, a well characterised and relatively simple technique which has proved invaluable in the expression and purification of many eukaryotic derived proteins (see Carrol and Laughon (1987) for a review of the technique and its applications). This is advantageous since there are few *E.coli* proteins larger in size than β-galactosidase, and additionally, in many instances the fusing of the foreign protein to β-galactosidase can lead to considerable increases in stability of the foreign portion, greatly increasing intracellular yields. The resulting fusion can usually be purified by a variety of relatively simple procedures to give an antigen of high purity. Such procedures make specific use of the β-galactosidase moiety of the resulting fusion, and can include affinity chromatography to bind the β-galactosidase segment (Ullmann 1984). Simple purification procedures making use of the uniquely large size of the resulting protein also exist. This can include SDS-polyacrylamide gel electrophoresis, ammonium acetate precipitation, or gel filtration. A further advantage, although not one of importance in this application of the technique, is that fusion to β-galactosidase

provides a convenient assay with which to monitor purification of a protein. In other circumstances, purification of an identified but as yet uncharacterised protein with no assignable activity would be essentially impossible.

To construct the β-galactosidase-DnaA fusion protein described in this study it was decided to use the pUR series of expression vectors constructed by Ruther and Muller-Hill (1983). These are small multi-copy expression vectors using the pMB1 replication origin, and contain a modified *E.coli lacZ* gene under control of the *lacUV5* promoter, such that expression of the constructed gene fusion is easily achieved upon addition of isopropyl β-D-thiogalactopyranoside (IPTG) to the growth media. Expression of *lacZ* is repressed by maintaining these plasmids in a host which overproduces lac repressor due to the $lacI^q$ mutant allele. The carboxyl-terminal of the β-galactosidase gene in these plasmids has been modified to introduce a polylinker into which foreign DNA fragments can be cloned; these cloning sites exist in all three reading frames. The selectable marker for these plasmids is ampicillin resistance.

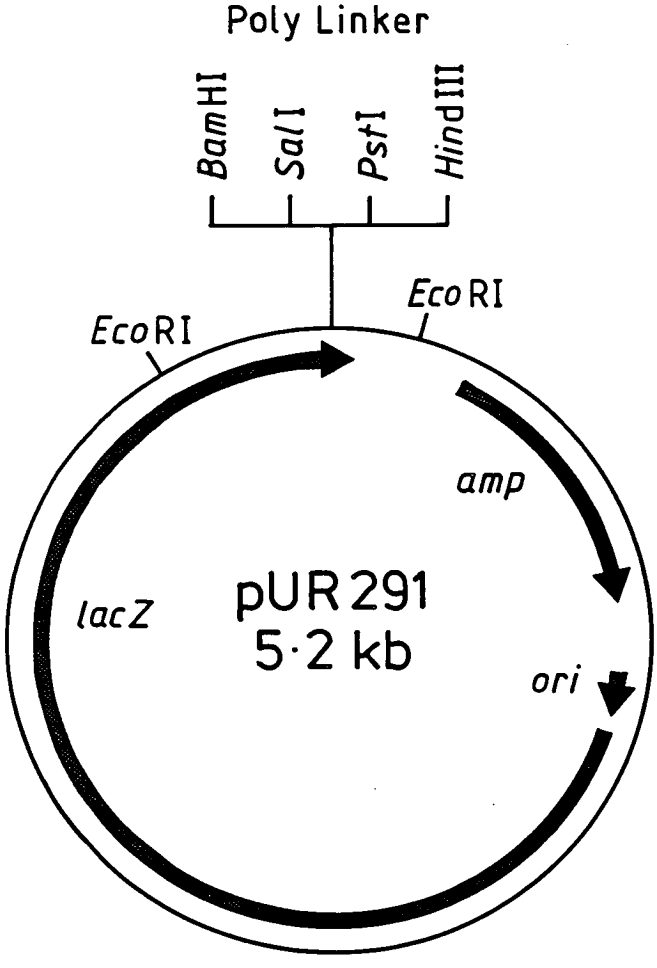
Two additional vector systems for the expression of cloned genes as part of a β-galactosidase fusion protein in *E.coli* have been described. The vectors described are the λgt11 system developed by Young and Davis (1983a, 1983b), and the pEX plasmid-based system of Stanley and Luzio (1984). Both systems were considered here, but rejected in favour of the pUR system described above. The λgt11 vector tends to be used more for the isolation of genes by using antibody probes to screen cDNA libraries rather than as an expression system *per se*. Its large size in comparison to the plasmid-based systems would tend to make cloning experiments unnecessarily complicated, and perhaps most importantly with regard to its suitability as an expression vector, the cloning sites are in the middle of the *lacZ* gene rather than at the carboxyl-terminus as per the plasmid-based systems. Successful cloning of the insert fragment usually results in the loss of β-galactosidase activity then, precluding a subsequent purification by affinity chromatography. The pEX expression vectors, in common with the pUR-series generally retain their β-galactosidase activity upon creation of the gene fusion. The pEX vectors are fundamentally very similar to the pUR-series, except that expression of the gene fusion is under control of the λ P_R promoter rather than *lacUV5*, and induction is caused by thermal inactivation of the λ cI^{857} repressor protein rather than addition of IPTG. Apart from this, the vectors are essentially identical. It was decided to use the pUR-series of vectors in preference over the pEX vectors for the simple reason that transformation using these latter plamids is inherently more difficult due to the requirement to perform the heat-shock

step at lower temperatures than is optimum because of the *ts* phenotype of the cI^{857} protein. For this reason use of the pUR vectors was preferred.

5.2.2 Choice of cloning strategy; use of a synthetic oligonucleotide

The first task in the construction of a *dnaA-lacZ* gene fusion in one of the pUR-series of vectors was to identify suitable restriction endonuclease sites which

FIGURE 5.3 Restriction map of pUR291



Restriction map of vector pUR291 showing polylinker and the direction of transcription of the lacZ gene. Re-drawn from Ruther and Muller-Hill (1983).

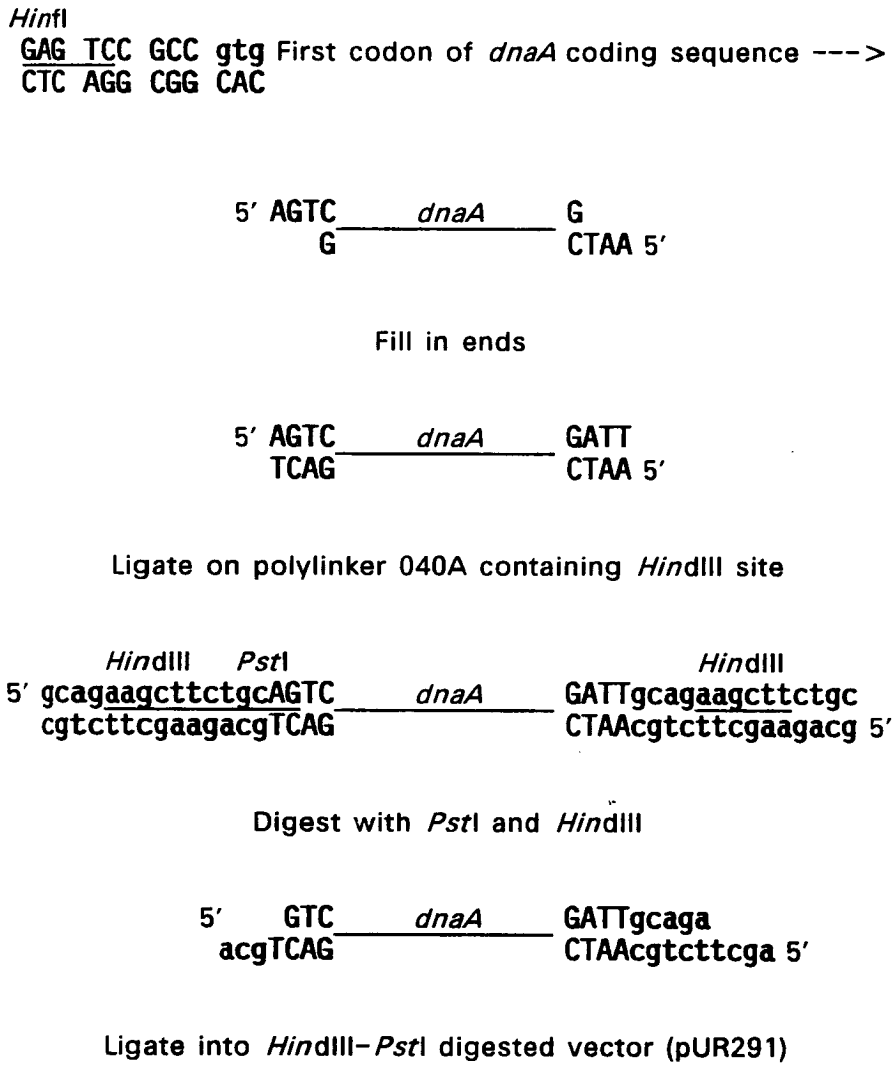
could be used to cut both vector and insert DNA's. The polylinker in the pUR-vectors contains unique cloning sites for the enzymes *Bam*HI, *Sal*I, *Pst*I, and *Hin*dIII (figure 5.3). Ideally then, it would be desirable to cut out the *dnaA* gene on a fragment contained within one or more of these restriction sites. Such perfect situations rarely arise in practice, and in fact none of these restriction enzymes proved suitable for the isolation of the *dnaA* gene. The most suitable restriction enzyme with which to remove the *dnaA* coding sequence in fact appeared to be *Hin*fI, which recognises the nucleotide sequence G'ANTC. The sites in relation to the *dnaA* gene are shown in figure 5.2, and the resulting fragment includes the entire *dnaA* coding sequence minus the promoter region; the *Hin*fI site being only 8bp upstream of the beginning of the coding sequence (Hansen *et al.* 1982; see also figure 5.4). The resulting fragment (cut from pJM87) could easily be isolated by agarose gel electrophoresis prior to elution from the gel and subsequent cloning steps (see figure 5.1).

Regarding the cloning of this fragment into the pUR vector, two options presented themselves. Since the polylinker in the vector does not contain suitable *Hin*fI cloning sites, the protruding 5' ends of the fragments would have to be filled in prior to either a blunt-ended ligation step or the addition of synthetic linkers. Two apparent disadvantages of a simple blunt-end ligation exist. The first is the low efficiency of ligation achieved with blunt-ended fragments , and the fact that recircularisation of the vector would probably occur at a high frequency. This could be reduced by de-phosphorylation of the vector fragment, but this carries the risk of removing extra bases and thus introducing frame-shift mutations into the resulting gene fusion. An additional minor disadvantage is that the insert fragment could be cloned in either orientation with equal efficiency; additional screening of recombinant plasmids would therefore be necessary.

The second option was to make use of the slightly different terminii resulting from the *Hin*fI digestion of the *dnaA* fragment and ligate on synthetic oligonucleotides containing restriction enzyme sites. The strategy adopted is shown in figure 5.4. Since the middle base of the *Hin*fI recognition sequence is variable, digestion and filling-in of the protuding 5' ends results in a different sequence to which the linker is added. Synthesis of the appropriate oligonucleotide (as shown in figure 5.4) enabled the *dnaA* gene to be contained within a *Pst*I - *Hin*dIII restriction fragment, which could then be cloned directly into the similarly restricted vector. This alleviated the problem of vector recircularisation, and additionally introduced new cloning sites which could be used at a later date should further isolation and cloning of the *dnaA* gene be required. This fragment could then be cloned into the vector pUR291 to maintain the

correct reading frame of the *lacZ-dnaA* gene fusion.

FIGURE 5.4 Cloning strategy using 040A polylinker



5.2.3 Construction of pJM88

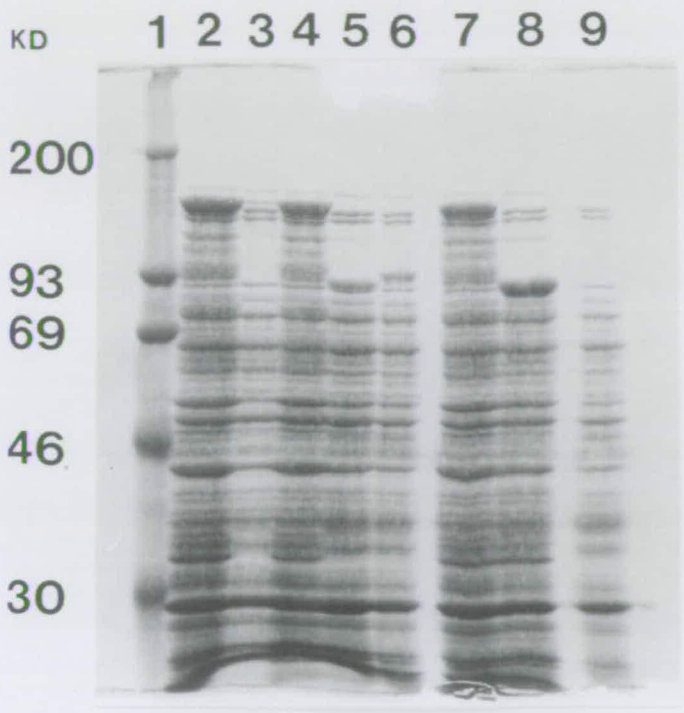
To construct the *dnaA-lacZ* gene fusion, plasmid pJM87 was first digested with *Hin*fI and the fragments separated on an agarose gel. The appropriate fragment was excised from the gel, electroeluted using the biotrap apparatus, the ends were filled in using the Klenow fragment of DNA polymerase I, and finally the polylinker 040A (figure 5.4) was blunt-end ligated on. The resulting fragment was then digested with *Hin*dIII and *Pst*I, and the linker fragments separated by agarose gel

electrophoresis followed by excision and subsequent purification of the *dnaA* band. Similarly, the vector was digested with *Pst*I and *Hin*dIII, and the appropriate fragment excised from an agarose gel. The vector and insert fragments, both complete with *Pst*I and *Hin*dIII sticky ends were then ligated together and subsequently used for transformation.

Initially it was attempted to transform the ligation mixture into strain BMH71-18, the usual host strain for such β-galactosidase fusion plasmids. This contains the *lacI*^{q} allele on an F prime plasmid to minimise expression of the plasmid-based *lacZ* gene. Transformation of the ligation mix directly into this strain failed to yield any recombinants however, suggesting perhaps that suppression of *lacZ* expression might not be total, and that a basal level of expression might be occurring. the resulting fusion protein was deleterious to the cell, this might explain the failure to isolate transformants in this strain. As a precaution it was decided to transform the ligation into strain CB831, which contains the pMB1-compatible plasmid pVH1. This plasmid also contains the *lacI*^{q} allele, but since it is present in a much higher copy number than the unit-copy F prime, repression should be much more efficient.

The ligation mix was used to transform this strain then, and the mixture plated at 37°C on amp50 plates. Several colonies were obtained, and exhibited a slightly mottled appearance in comparison to the pUR291 transformed controls. The colonies were streaked to purity and grown up in liquid media prior to the addition of IPTG. An SDS-polyacrylamide gel showing the high molecular weight proteins of these cultures is shown in figure 5.5. It is apparent that several of these strains contain a high molecular weight protein of the anticipated size of the β-galactosidase-DnaA fusion. The constructed plasmid was called pJM88, and was subsequently used to transform BMH 71-18. Transformants were obtained, but exhibited a low viability. This transformed strain was subsequently used to prepare a stock of pJM88 DNA, since CB831 pJM88 also contained plasmid pVH1 (which is similar in size to pJM88). A gel showing restricted pJM88 DNA, together with the parentals pJM87-01 and pUR291 is shown in figure 5.6, and a restriction map of pJM88 is shown in figure 5.7.

FIGURE 5.5 DnaA-β-galactosidase fusion protein in CB831



Coomassie blue stained whole cell extract; 8% linear gel

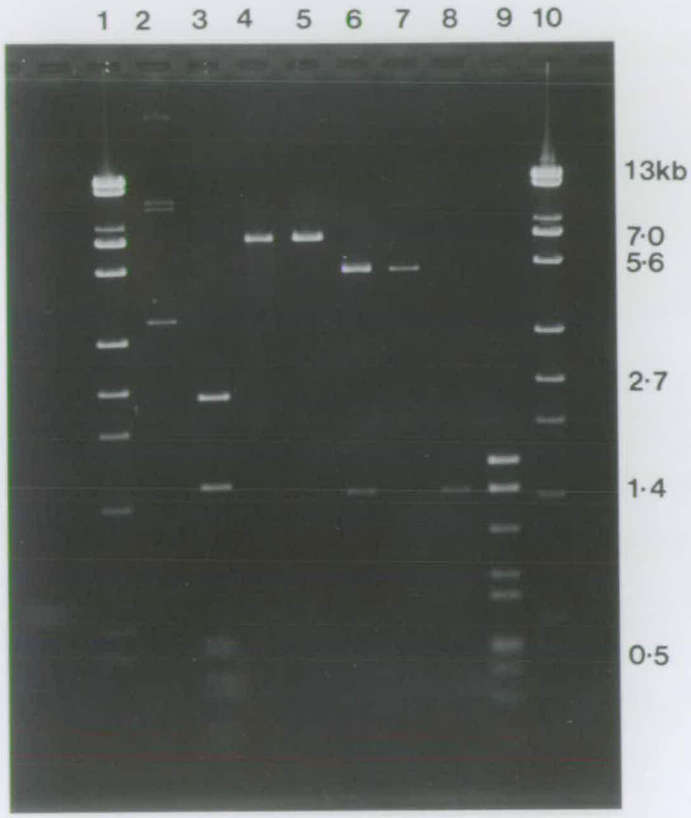
Track 1: Molecular weight markers: Myosin, Phosphorylase b, Bovin Serum Albumin, Ovalbumin, Carbonic Anhyrdase

Tracks 2-7: CB831 clones containing potential fusion plasmid. Fusion protein can be seen in tracks 2, 4 and 7

Track 8: CB831 pUR291. The lower molecular weight β-galactosidase protein is clearly visible

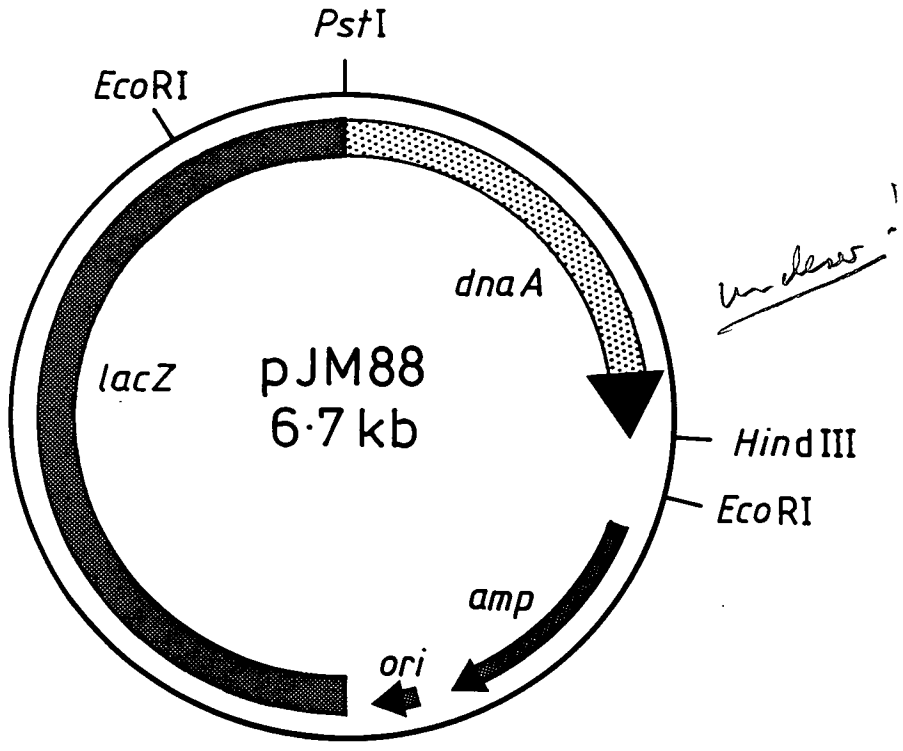
Track 9: CB831 control

FIGURE 5.6 Restriction analysis of pJM88



Track 1: λcI^{857} AccI size standards
Track 2: pJM88 undigested
Track 3: pJM88 HinfI
Track 4: pJM88 PstI
Track 5: pJM88 HindIII
Track 6: pJM88 PstI–HindIII
Track 7: pUR291 PstI–HindIII
Track 8: HinfI dnaA fragment purified from gel
Track 9: pJM87-01 HinfI
Track 10: λcI^{857} AccI size standards

FIGURE 5.7 Restriction map of pJM88



lacZ DNA marked in block; dnaA DNA marked in hatch. Notice that the dnaA gene is contained within a 1.5kb PstI–HindIII fragment.

5.3 Effect of DnaA–β–galactosidase fusion protein on cell physiology

Bearing in mind the difficulties in transforming pJM88 into BMH71–18, it was decided to investigate the effect of induction of the fusion protein on cell physiology. Strains BMH71-18 pJM88 and CB831 pJM88 were streaked out on plates plus and minus the inducer IPTG. Both strains containing pBR328 were included as controls. The plates contained ampicillin at 50μgml^{-1} and were incubated at 37°C and growth compared the following day. The results are shown in table 5.7, and it would appear that induction of the β-galactosidase-DnaA fusion results in a killing of the culture. The reduced growth caused by pJM88 even in the absence of IPTG would tend to suggest that repression by the *lacI*^{q} product is only partial in both instances.

TABLE 5.7 Effect of pJM88 in CB831 and BMH71-18

STRAIN	PLASMID	LA	LA+IPTG
COLONY DIAMETER (MM)			
BMH71-18	pJM88	0.3	-
	pBR328	1.5	1.5
CB831	pJM88	0.5	-
	pBR328	1.5	1.5

L-Agar plates containing amp50. Plates incubated overnight (c. 18hrs) at 37°C.

The effect of induction upon cell size was then investigated. Cultures of CB831 pJM88 and BMH71-18 pJM88, together with control cultures (containing pBR328) were grown in L-broth + amp50 and IPTG added to induce expression. After two hours the cell sizes were measured in a Coulter Counter, and as can be seen in table 5.8, in comparison to the controls induction of the β-galactosidase-DnaA fusion protein results in filamentation of the culture.

TABLE 5.8 Induction of fusion protein; effect on cell size

STRAIN	PLASMID	LB	LB+IPTG
CELL SIZE (MEDIAN)			
BMH71-18	pJM88	19	29
	pBR328	17	17
CB831	pJM88	19	27
	pBR328	19	19

Cultures grown in L-Broth amp50.

How might this induction result in filamentation? Two possibilities appeared likely. Firstly, the presence of aberrant and abnormal proteins (such as this fusion for example) can result in induction of the heat shock response (Goff and Goldberg 1985, VanBogelen *et al.* 1987), which causes a transient cessation of cell division and consequent filamentation (Tscuchido *et al.* 1986). A second possibility is that the β-galactosidase-DnaA fusion protein might be specifically interfering with the initiation of DNA replication via the interaction of DnaA at *oriC*.

To investigate this, a culture of BMH71-18 pJM88 (37°C) was induced for expression of the fusion, and the cells examined for DNA content after growth for 2 hours. The technique of whole-cell fluorimetry was used for this. As a comparative control, the *dnaA46ts* strain MM185 was grown and shifted from 30°C to 40°C, followed by maintenance at this higher temperature for two hours after which the cells were examined for DNA content. The *dnaAts* culture was comprised of mostly long uninucleate filaments, with a considerable number of cell 'ghosts'. In contrast, the BMH71-18 pJM88 culture exhibited only relatively mild filamentation, with less DNA than might be expected for a normal culture but certainly more than was present in the *dnaAts* strain at the non-permissive temperature (data not shown).

This tended to suggest that the β-galactosidase-DnaA fusion might not specifically affect DNA replication, although the possiblity that the fusion exhibited a 'leaky' phenotypic effect on replication could not be excluded. It was decided to transform pJM88 into MM185 (*dnaA46ts*) and induce expression at 30°C and 40°C to see if the fusion protein was able to complement a *dnaAts* mutation. Transformants were obtained at 30°C, and then streaked at 30°C and 40°C, plus and minus the inducer IPTG. MM185 pBR325 was included as a control culture. The results are shown in table 5.9. They demonstrate that the β-galactosidase-DnaA fusion protein is unable to complement the *dnaA46ts* mutation, but rather suprisingly, induction at 30°C did not appear to seriously affect either cell growth or viability. At this point it was realised that all induction prior to this had occurred at 37°C; possibly the deleterious effect of the fusion protein only became apparent at higher temperatures.

It was decided to induce the fusion protein in strain TP8511 pJM88 (a *lac* delete strain; see section 5.4) at a variety of temperatures and compare the effect on cell growth and viability. TP8511 pBR328 was included a a control. Additionally, it was decided to investigate whether the deleterious effect of the fusion was due to interference with initiation at *oriC*, or whether a more general effect on cell physiology might be responsible. To do this, pJM88 was transformed into strain CM1843, which is

deleted for *oriC* and instead replicates from a DnaA-independent integrated R1 plasmid origin. If the growth defect was due to a specific interaction at *oriC* then this effect should not be present following induction in this strain.

TABLE 5.9 Testing pJM88 for the ability to complement a *dnaAts* mutation

STRAIN	PLASMID	30°C LA	30°C LA+IPTG	40°C LA	40°C LA+IPTG
COLONY DIAMETER (mm)					
MM19 *dnaA46*	pBR328	1.0	1.0	-	-
	pJM88	0.8	0.8	-	-

Plates contained amp50 to select for plasmids, and were incubated overnight (c. 18hrs) whereupon colony size was measured.

The results of these experiments are shown in table 5.10. They clearly demonstrate that the deleterious effect of fusion protein induction is a temperature dependent process; indeed, at 30°C no significant effect upon cell growth is apparent. Additionally, it would appear that the effect is not due to a specific disruption of DNA replication from *oriC*, since it is seen in *oriC* delete strains, although this does not of course rule out the possibility of this being an additional influence. Again, the observed reduction in growth following induction of the fusion is a temperature dependent effect.

Why should induction of the fusion protein prevent cell growth only at higher temperatures while not at 30°C? The most likely explanation would appear to be that the fusion protein denatures and forms aggregates/precipitates at the higher temperature, disrupting cellular processes and inducing the heat-shock response (for a general review of the heat-shock response see Neidhardt and VanBogelen 1987). Recently a paper by Gaylo *et al.* (1987) has reported the construction of a similar β-galactosidase-DnaA fusion protein. This protein appeared to be able to complement a *dnaAts* mutation both in host cell and plasmid DNA replication, in contrast to the results reported here. Similarly, induction of this fusion at high temperatures did not

TABLE 5.10 Effect of temperature on pJM88-containing cultures

STRAIN	PLASMID	30°C	40°C
COLONY DIAMETER (mm)			
TP8511 *dnaA*$^+$	pBR328	1.0	1.5
	pJM88	0.8	-
CM1843 Δ*oriC*	pBR328	0.5	0.5
	pJM88	0.5	-

Cultures were streaked to single colonies on LAamp50 plates and incubated overnight (c. 18hrs) at the appropriate temperature, whereupon colony diameter was measured.

lead to the cell death which has been reported in this study. However, it is possible to perhaps reconcile these differences if the construction details of the fusions are considered. The fusion protein described in this work consisted of the N-terminus of DnaA fused to the C-terminus of β-galactosidase The construct of Gaylo *et al.* consisted of the C-terminus of DnaA fused to the N-terminus of β-galactosidase, with a short collagen linker molecule separating the two proteins. (This construct was under control of the λ P_L promoter rather than the *lacUV5* promoter of the pJM88 construct). Additionally, the authors could not rule out the possibility that proteolytic cleavage of the fusion protein at this collagen linker *in vivo* might be occurring to produce a biologically active DnaA fragment.

5.4 Purification of the DnaA-β-galactosidase fusion protein prior to immunisation

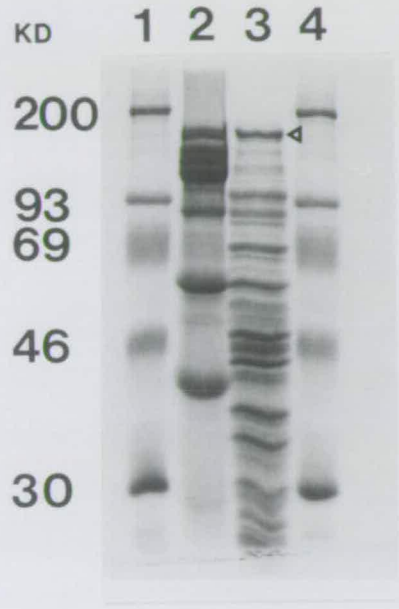
It was initially decided to purify the β-galactosidase-DnaA fusion protein on the basis of affinity chromatography, since this method has been reported to result in a preparation of greater than 95% purity (Ullmann 1984). Further purification on the basis of size by SDS-polyacrylamide gel electrophoresis could then be used to remove additional contaminants whilst at the same time lending a visual check to the purity of the preparation. For this to be successful would obviously require that the fusion protein possess β-galactosidase activity, and this was examined by transforming pJM88 into TP8511, inducing expression with IPTG, and performing β-galactosidase assays to look for activity. The plasmid was transformed into TP8511 since this strain is *lac* delete and *recA*. The strains into which pJM88 had already been transformed

were either *rec*$^+$ (BMH71-18), and therefore liable to gene rearrangements, or else *lac*$^+$ (CB831), and therefore supplying a chromosomally encoded β-galactosidase protein which would obviously be purified along with the fusion. When assays were performed on an induced culture of TP8511 pJM88, relatively high levels of β-galactosidase activity were obtained, suggesting that the fusion protein did indeed possess β-galactosidase activity, although the possibility that activity of a breakdown product rather than the intact fusion was being monitored could not be discounted.

An affinity column was prepared according to the method of Ullmann (1984). The fusion protein was induced in a culture of TP8511 pJM88 (2 litres), and purified by affinity chromatography using the protocol suggested by Ullmann (1984). The purified protein was eluted from the column and found to retain 80% of the total β-galactosidase activity of the original sample as measured at the time of loading. A sample of this eluate was run on an SDS-polyacrylamide gel alongside a whole cell extract of induced TP8511 pJM88. This is shown in figure 5.8. It is clear that many breakdown products and/or contaminating proteins are present. Additionally, it would appear that a band corresponding to the intact fusion protein does not appear to be present, leading onto the possibility that the intact protein may not in fact possess β-galactosidase activity. Examining the gel showing the protein products in CB831 pJM88 alongside CB831 pBR328 (figure 5.8), clearly shows the presence of large molecular wieght protein bands presumably due to breakdown of the fusion protein in the former. Thus it is possible that the intact fusion protein might not in fact possess any β-galactosidase activity, and thus breakdown products alone would be purified using this approach. Alternatively, it is possible that the intact fusion protein was bound to the column, but degradation occurred during or after this stage.

Since it was unclear which protein band corresponded to the largest fusion protein fragment, it was considered unwise to purify one of these fragments from the gel to use for antibody preparation. Instead it was considered adaquete to cut the intact fusion protein band from an SDS-polyacrylamide gel of a whole cell extract and use this for immunisation and antibody production. Although contamination by small amounts of similarly sized *E.coli* proteins might be expected (e.g. the β and β' subunits of RNA polymerase) in a Western blotting detection system this was considered acceptable. Since DnaA has an apparent molecular weight of 48-54kd in an SDS-polyacrylamide gel (Hansen and von Meyenburg (1979), Yuasa and Sakakibara 1980), any contaminating antibodies additional to DnaA would detect proteins in the molecular weight range 150-180kd, and consequently would not interfere with visualisation of the very much smaller DnaA protein and its breakdown products.

FIGURE 5.8 SDS-polyacrylamide gel electrophoresis of affinity purified fusion protein



Coomassie blue stained whole cell extract; 8% linear gel. The intact fusion protein band in TP8511 is marked with an arrowhead.

Track 1: Molecular weight markers: Myosin, Phosphorylase b, Bovin Serum Albumin, Ovalbumin, Carbonic Anhyrdase

Track 2: Affinity purified DnaA-β-galactosidase fusion protein

Track 3: TP8511 pJM88 whole cell proteins

Track 4: Molecular weight markers

The earlier result concerning the temperature-dependent nature of cell killing by the fusion protein was not discovered until after this investigation into affinity chromatography. It may be possible that induction at 30°C (where the fusion does not cause cell killing) might result in less breakdown of the protein *in vivo*, leading to a more efficient purification by this procedure. However, it would appear that some proteins are evidently less stable as part of a β-galactosidase fusion than others, and this probably affects the suitability of this technique as a means of purification (for examples of the use of fusion proteins in *E.coli* see Lim *et al.* (1987), Majumdar *et al.* (1987), Ruther *et al.* (1982), and Wilken-Bergmann *et al.* (1983)).

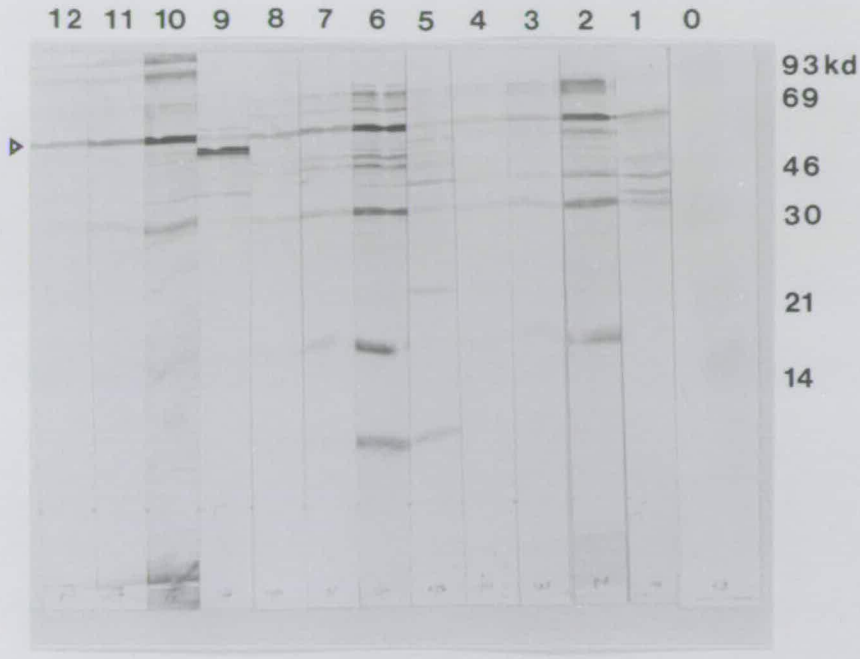
To prepare the antigen for innoculation then, the fusion protein was cut from a gel, and electroeluted into Tris-Glycine-SDS gel buffer (see materials and methods) using the BioTrap™ apparatus. Despite the fact that a single unique band of 170kd had been excised from the gel, when an aliquot of this electroeluted protein was run on an SDS-polyacyrlamide gel, a ladder of breakdown fragments (170kd-40kd) was seen on the gel, revealing the inherent instability of this fusion (data not shown). This preparation was then used to innoculate three rabbits following the immunisation schedule detailed in chapter 2.

5.5 Use of anti-DnaA antibodies in Western blotting

5.5.1 Titration of antibody

After the immunisation schedule was complete, the antisera obtained from the three rabbits were compared with the pre-immune sera for the presence of specific anti-DnaA activity. Additionally, the antisera were titrated to discover which dilution gave the highest signal to noise ratio. Whole cell extracts of the *dnaA*$^+$ strain MM18 were run on an SDS-polyacrylamide minigel and electroblotted onto nitrocellulose filters. Pre-stained molecular weight markers were included at either side of the gel. The filter was then cut into strips, and used to titrate the antisera by incubating with dilutions of 1:100, 1:800 and 1:2000. Additionally, a filter strip was incubated with the pre-immune sera from each rabbit diluted 1:500 as a control. The results of this experiment are shown in figure 5.9. It is clear that a strong band of 50-60kd is present in each antiserum preparation, while being very much reduced in the pre-immune sera. Since the filters had been washed together, it was considered possible that cross-hybridisation during this stage might have occurred. However, it seemed quite clear that a strong band of 50-60kd was now being visualised by

FIGURE 5.9 Titration of antisera



The position of the strong 50-60kd molecular weight antigen is marked with an arrowhead. 14% linear gel.

Track 0: Molecular weight markers: Myosin, Phosphorylase b, Bovin Serum Albumin, Ovalbumin, Carbonic Anhyrdase, Trypsin Inhibitor.
Track 1: Rabbit 150 pre-immune serum 1:500 dilution
Track 2: Rabbit 150 antiserum 1:100 dilution
Track 3: Rabbit 150 antiserum 1:800 dilution
Track 4: Rabbit 150 antiserum 1:2000 dilution
Track 5: Rabbit 151 pre-immune serum 1:500 dilution
Track 6: Rabbit 151 antiserum 1:100 dilution
Track 7: Rabbit 151 antiserum 1:800 dilution
Track 8: Rabbit 151 antiserum 1:2000 dilution
Track 9: Rabbit 152 pre-immune serum 1:500 dilution
Track 10: Rabbit 152 antiserum 1:100 dilution
Track 11: Rabbit 152 antiserum 1:800 dilution
Track 12: Rabbit 152 antiserum 1:2000 dilution

the putative anti-DnaA serum, especially since the dilution of pre-immune antisera which resulted in this faint band (1:500) was much less than the 1:2000 dilution of challenged sera which gave a much stronger band. It also seemed apparent that a dilution of 1:2000 gave the best signal with the the lowest background. Interestingly enough, the serum which appeared to give the cleanest results (rabbit no. 152) exhibited activity against a lower molecular weight protein in the pre-immune sera, suggesting that the animal had been exposed to an *E.coli* cross-reacting antigen prior to immunisation. It was considered possible that this might perhaps explain the better response of this animal when challenged with the fusion protein.

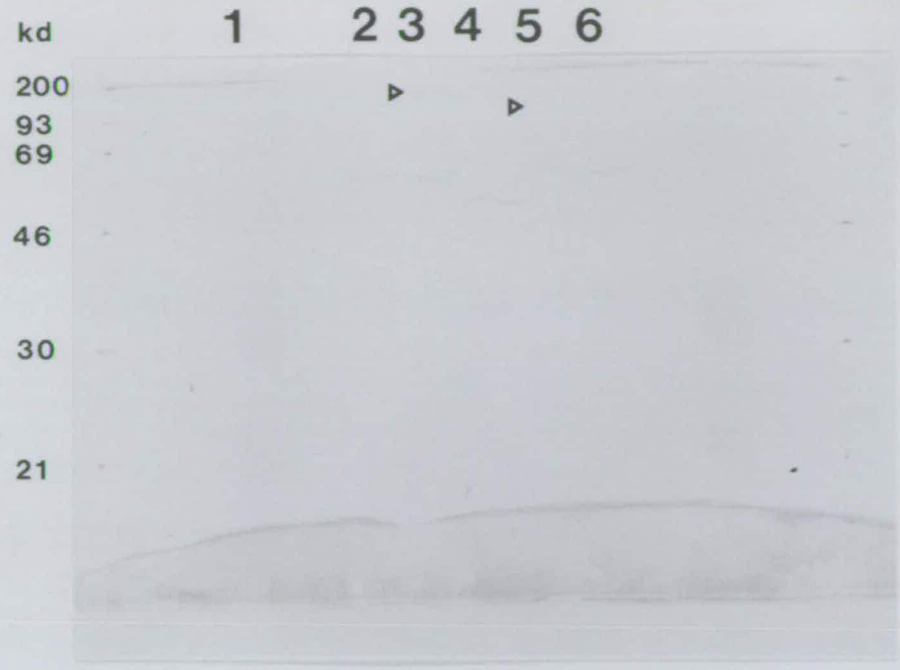
5.5.2 Visualisation and determination of levels of the DnaA protein

Since antiserum number 152 appeared to give the best signal to noise ratio, it was decided to use this preparation in further experiments, and proceed with an investigation into whether it possessed anti-DnaA activity (i.e. is the strong band which reacts with the antiserum of animal 152 the DnaA protein?). To investigate this, a further gel was run and electrolotted onto nitrocellulose, followed by incubation with a 1:2000 dilution of antisera 152. The results of this are shown in figure 5.10, and the different tracks correspond to a whole cell extract from strain DK249 (a *dnaA* null mutant); TP8511; TP8511 pJM88 induced for fusion protein expression; 90% pure DnaA protein (obtained from E. Wahle); and a *dnaA*$^+$ control culture of MM18.

At first glance the results seem difficult to interpret. It is clear that the antiserum contains anti-DnaA activity, since a band corresponding to the fusion protein (and its breakdown products) is apparent in TP8511 pJM88, while at the same time a band in the purified DnaA protein track is seen also. Additionally, a faint band corresponding to the β-galactosidase protein is present in the MM18 track. However, the major presumed DnaA protein band observed in the previous blot appears to be of a larger molecular weight than the purified DnaA protein. Additionally, this band is also present in the supposed *dnaA* null mutant DK249. Was it possible that this strain is really *dnaA*$^+$, and that the purified DnaA protein is smaller than the protein *in vivo*? Or does the antiserum contain antibodies to an additional protein besides DnaA and β-galactosidase?

To help resolve this, the blot shown in figure 5.11 was made. This contained whole cell extracts from 3 different *dnaA* null mutants (DK249, EH3791, EH3821), so it was thought exceedingly unlikely that all 3 could have reverted to *dnaA*$^+$. Also included were tracks from TP8511 pJM88 (fusion protein), 2 tracks from an induced

FIGURE 5.10 Western Blot of purified DnaA protein



The faint band corresponding to β-galactosidase in MM18, and the band corresponding to the DnaA-β-galactosidase fusion protein in TP8511 pJM88 are marked with arrowheads. 14% linear gel.

Track 1: Molecular weight markers: Myosin, Phosphorylase b, Bovin Serum Albumin, Ovalbumin, Carbonic Anhyrdase, Trypsin Inhibitor.

Track 2: TP8511

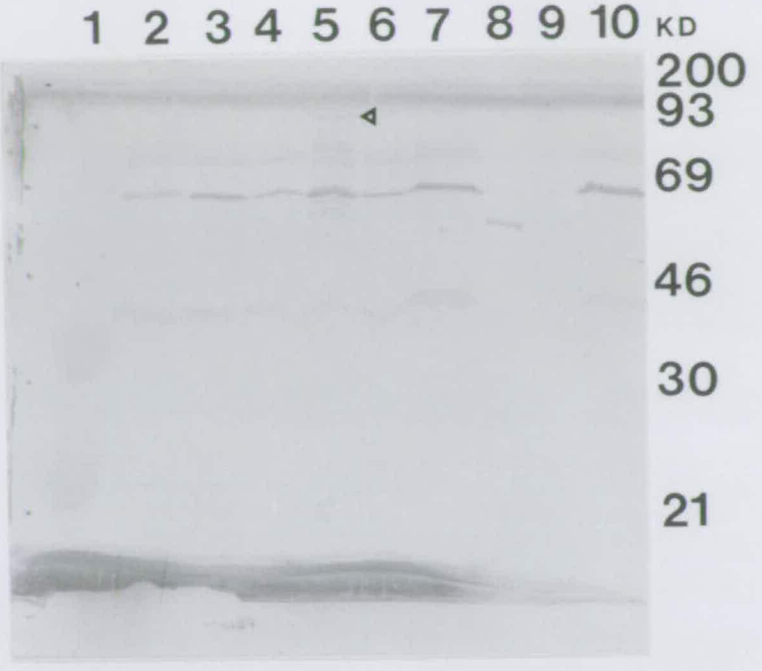
Track 3: TP8511 pJM88 induced for fusion protein expression

Track 4: Purified DnaA protein

Track 5: MM18 *dnaA*$^+$

Track 6: DK249 *dnaA* null mutant

FIGURE 5.11 Western Blot of *dnaA* null mutants



The faint band corresponding to the DnaA-β-galactosidase fusion protein in TP8511 pJM88 (track 5) is marked with an arrowhead. The purified DnaA protein band in track 10 can be seen on the original blot. 14% linear gel.

Track 1: Molecular weight markers: Myosin, Phosphorylase b, Bovin Serum Albumin, Ovalbumin, Carbonic Anhyrdase, Trypsin Inhibitor.

Track 2: DK249 dnaA null mutant

Track 3: EH3791 dnaA null mutant

Track 4: EH3821 dnaA null mutant

Track 5: TP8511 pJM88 (induced for fusion protein expression)

Track 6: TP8511 pBC32 (induced for dnaA over-expression); 3ul extract

Track 7: TP8511 pBC32 10ul extract

Track 8: Purified DnaA protein (40ng)

Track 9: Purified DnaA protein (4ng)

Track 10: MM18 $dnaA^{+}$

TP8511 pBC32 (which over-expresses *dnaA* under control of P*lac*; 10 and 3µl loaded), 2 tracks of purified DnaA protein (40 and 4ng), and finally a *dnaA*$^+$ control, MM18. The strong band of a slightly larger molecular weight than purified DnaA appears to be present in all tracks, suggesting it does not in fact correspond to the DnaA protein. The intact fusion and breakdown products previously observed with TP8511 pM88 are seen here also, suggesting a considerable amount of proteolytic degradation *in vivo*. Interestingly enough, one of the breakdown bands appears to run at the same position as the purified DnaA protein, leading onto the possibility that proteolytic cleavage at or near the fusion junction may be occurring. An alternative explanation might be that the presence of the fusion protein may be resulting in an increased level of synthesis of the chromosomally-encoded DnaA, for example if autorepression of *dnaA* was affected. A faint band corresponding to the purified DnaA protein is just visible in the heavily loaded DnaA over-producing strain pBC32, but only in the more heavily loaded track. In a normal *dnaA*$^+$ strain this band cannot be seen. Since this antiserum can be used to visualise amounts of DnaA protein as little as 4ng (roughly at about the limits of detection in a Western blotting system), this would tend to suggest that the intracellular amounts of DnaA protein are very low under normal conditions. Comparing the strength of bands seen in this blot, it is in fact possible to calculate an upper limit for the amount of DnaA protein in an individual cell. For example:-

Amount of DnaA protein in MM18 (*dnaA*$^+$) track > 4ng then

Taking 4ng as an upper limit;

No. moles DnaA present = $\frac{4 \times 10^{-9}}{52.5 \times 10^{3}} = 7.6 \times 10^{-14}$

No. molecules DnaA = 7.6×10^{-14} x Avogadro's no. (6×10^{23})

$= 4.8 \times 10^{10}$ molecules

Amount of cell extract loaded on gel (MM18) = 0.5ml of an $O.D._{540} = 0.2$

= c. 5×10^{7} cells

Therefore no. molecules DnaA per cell > $\frac{4.8 \times 10^{10}}{5 \times 10^{7}}$

> 1000 molecules DnaA per cell

This is roughly comparable with the estimates of other researchers (Judy Zyskind, pers comm. to M. Masters; Sekimizu *et al.* 1988, Sakakibara and Yuasa 1982).

Interestingly enough, the DnaA band visualised in the over-producing strain TP8511 pBC32 is only about 2-3 times as strong as that of the 4ng purified DnaA protein, which would suggest that even in an overproducing strain induction of *dnaA* expression is not particularly efficient (this in fact is in agreement with the findings of Xu and Bremer (1988) who found that overproduction of DnaA using this system was not very efficient).

The level of DnaA protein in the cell tends to suggest that the use of unpurified antiserum is probably not sensitive enough to detect DnaA in the cell in a Western blotting system. A similar situation has been reported by another investigator who has been attempting to use anti-DnaA antibodies to measure DnaA protein levels in a Western blotting system (Patrick Hughes pers. comm.). In this instance it proved possible to visualise the DnaA protein in a Western blot, but only after partial purification prior to electrophoresis. This is obviously undesirable if quantitative measurements of protein levels are to be made, and additionally, could lead to the presence of breakdown products being overlooked. Unfortunately then, it seems that although anti-DnaA antibodies were successfully obtained, they appeared unable to help in answering the question of how *groE* might suppress *dnaAts* under the detection system chosen. Purification of the antibodies should help in this respect, but only if a more sensitive detection system (such as radiolabelled *Staphylococcal* protein A for example) was adopted. In this instance the sensitivity of the detection system (immunogold silver staining) appears to be the limiting factor (1ng of protein being the limit of the detection). The use of affinity purified antibodies to DnaA should allow incubation at a lower dilution and thus hopefully increase sensitivity. If time and experimental requirements had been available this would have been the obvious next step regarding use of the antiserum. It is possible that more sample could be loaded onto a gel, thus increasing the overall DnaA level available for detection, although this would also probably increase the background binding. Additionally, the capacity of the polyacrylamide gel tends to be the limiting factor here, with overloading of the gel resulting in smearing and poor resolution. The most promising approach would probably be to use a more sensitive detection system than the immunogold-silver staining technique employed here. The Kornberg laboratory have reported detection of DnaA protein in a whole cell extract using Western Blotting (Sekimizu *et al.* 1988), using affinity purified antibodies in conjunction with a radiolabelled detection system. Additionally, it would appear that it is possible to detect the DnaA protein in a two-dimensional gel system using anti-DnaA antibodies with I^{125}-labelled *Staphylococcal* protein A as a detection system (Judy Zyskind, pers. comm. to M. Masters), and further investigation into the use of the available DnaA antisera may

yet prove fruitful.

5.5.3 The antiserum appears to possess anti-GroE activity

One last question concerning use of the anti-DnaA antibodies perhaps deserves consideration before this section is closed. That is, what is the strong protein band of c.60kd visualised in addition to DnaA and β-galactosidase by this antisera? The blot shown in figure 5.12 may perhaps provide an answer. This blot was made before it became apparent that the strongly detected protein band was not in fact DnaA. It was decided to examine the DnaA levels in a variety of *dnaAts* and *dnaA*$^+$ strains, in the presence and absence of pND5 at 30°C and 42°C. It was hoped this might show how the presence of extra GroE affected Dna protein levels and stability at the higher temperature. Examining this blot it is apparent that the levels of this strong 60kd band seem to vary from lane to lane, in a predictable way; that is, the higher the temperature, the greater the band intensity, and additionally, strains containing pND5 show much greater intensity of staining than strains without. Could this 60kd protein be GroEL? Certainly GroEL levels would be expected to be higher with an increase in temperature as is observed here, and additionally, the fact that this band is much stronger in pND5-containing strains would suggest that a plasmid-encoded protein is being expressed. Examination of figure 3.7 showing pND5 encoded proteins reveals no other protein of this molecular weight apart from GroEL. The strongest evidence that this protein is indeed GroEL probably comes from the very intensity of the band in pND5-containing strains in figure 5.12, there being so much protein present that the band is evidently overloaded and distorted; exactly as seen for the whole cell extract of a pND5-containing strain seen in figure 3.7. No other protein band is even remotely close in intensity. Thus it would seem highly probable that GroEL is indeed the protein being visualised in this instance.

If this band is indeed GroEL, as would seem likely, why should it be detected by these antisera? It was noted that this band could been seen very faintly using the pre-immune sera of all three rabbits (figure 5.9); it is possible that all three animals possessed antibodies to a GroEL-like protein prior to immunisation. It has recently become clear that GroE like proteins are present in a variety of bacterial species, and that the protein is very highly conserved, as well as being very immunogenic. The major antigenic determinant in a mycobacterial infection turns out to be a protein homologous to GroEL, and antibodies to this protein are cross-reactive to GroEL (Douglas Young pers. comm.; quoted in Hemmingsen *et al.* 1988). It would seem

FIGURE 5.12 Western Blotting of GroE over-producing strains

Equal amounts of cell extract were loaded onto each track. 14% linear gel.

Track 1: Molecular weight markers: Myosin, Phosphorylase b, Bovin Serum Albumin, Ovalbumin, Carbonic Anhyrdase, Trypsin Inhibitor.
Track 2: MM184 dnaA508 pND5 42°C
Track 3: MM184 dnaA508 pND5 30°C
Track 4: MM184 dnaA508 pBR325 42°C
Track 5: MM184 dnaA508 pBR325 30°C
Track 6: MM181 dnaA167 pND5 42°C
Track 7: MM181 dnaA167 pND5 30°C
Track 8: MM181 dnaA167 pBR325 42°C
Track 9: MM181 dnaA167 pBR325 30°C
Track 10: MM182 dnaA5 pND5 42°C
Track 11: MM182 dnaA5 pND5 30°C
Track 12: MM182 dnaA5 pBR325 42°C
Track 13: MM182 dnaA5 pBR325 30°C
Track 14: MM18 $dnaA^+$ pND5 42°C
Track 15: MM18 $dnaA^+$ pND5 30°C
Track 16: MM18 $dnaA^+$ pBR325 42°C
Track 17: MM18 $dnaA^+$ pBR325 30°C

plausible that each rabbit may have acquired antibodies to a GroE-type protein prior to immunisation, and these are now cross-reacting to GroEL. The presence of the faint band in the pre-immune sera would certainly agree with this. However, this may not be the entire answer, since if this were the total source of anti-GroEL antibodies, then why should the signal strength of this band be so much higher post-immunisation? This may in fact suggest an interaction between GroEL and DnaA such that GroEL was present in the inmmunising preparation, although it is difficult to see how this could have occurred, since the fusion protein was obtained from an SDS-polyacrylamide gel, and all protein types should be totally denatured.

5.6 Summary and discussion

The work presented in this chapter describes the cloning of the *dnaA* gene on a high-copy vector from a *Hind* III digested *E.coli* chromosomal library. This plasmid was subsequently used to construct a *lacZ-dnaA* gene fusion in a high-copy vector which results in the production of a 170kd protein which is lethal to the host strain when expressed at high temperatures. The fusion protein was purified and used to raise anti-DnaA antibodies. These antibodies appeared unable to visualise the DnaA protein in a normal cellular extract, suggesting that intracellular levels of this protein are very low (less than 1000 molecules per cell).

While attempting to clone the *dnaA* gene for this work, a variety of extrachromosomal suppressors of *dnaAts* were isolated. These fell into several size classes, and varied in the efficiency with which they were able to suppress. Some of them were able to suppress at 40°C immediately following transformation, however most of them appeared to suppress more efficiently if pre-grown at 30°C before temperature shift. Plasmid pHR3 also seemed able to mediate suppression of *dnaAts* mutants, although it tended to allow better growth at higher temperatures rather than increase the upper temperature at which a strain was able to survive. Over-expression of *rpoB* or *rpoC* might be responsible for the suppressiing effect of this plasmid.

Unfortunately it was not possible to use the anti-DnaA antibodies to provide an insight into the mechanism by which over-expression of the *groE* genes might act to suppress *dnaAts* mutations. The experiments described in the previous chapter had led to the conclusion that suppression was probably due to either a specific interaction between the two gene products, possibly at the origin of replication, or else that GroE might somehow effect the levels or stability of the DnaAts protein. With anti-DnaA antibodies it was hoped that it might be possible to see if both

proteins interacted *in vivo*; for example the *groE* gene product could be immunoprecipitated with monoclonal antibodies which were already available (Douglas Young pers. comm.), and this complex could then be probed with anti-DnaA antibodies to see if DnaA was coprecipitated. The finding that too little DnaA was present in the cell for it to be visualised without significant background problems effectively precluded such an approach. This was rendered even more unlikely when it was found that the antisera also appeared to show anti-GroE activity. In addition, it was hoped that DnaA protein levels could be estimated using Western blotting, which should also allow the visualisation of any breakdown products in a *dnaAts* strain under conditions of non-permissive growth. Again, the very low levels of DnaA protein in the cell effectively prevented the use of such an approach with the antisera available. Had time been available, affinity chromatography purification of the antibodies would have been attempted; together with a more sensitive detection system (I^{125}-labelled protein A) this might have allowed a more definitive investigation.

How might the use of antibodies to DnaA help in the investigation of suppression by GroE? Purification of the antibodies and a more sensitive detection system should certainly help, although it should be appreciated that a major problem is the low level of DnaA protein in comparison to all other cellular protein. Partial purification of the DnaA protein should help, although this would effectively rule out any quantitative investigation of DnaA levels. The use of monoclonal antibodies should result in far less problems with background, allowing greater concentrations of antibodies to be used. However, this would be a risky undertaking if using a fusion protein as the antigen, since a β-galactosidase epitope rather than a DnaA epitope may be the target. Additionally, DnaA breakdown products may not be efficiently visualised using such an approach. Purification of the polyclonal anti-DnaA antibodies might well provide the best route for a further immunological approach, hopefully reducing non-specific binding due to contaminating antibodies, and allowing an estimate of DnaAts protein levels under suppressing conditions.

CHAPTER 6

The phenotypic effects of over-expression of the *groE* genes

6.1 Introduction

The presence of the *groE* genes on a high copy-number plasmid (pND5) results in a number of phenotypic side effects to the host strain in addition to the suppression of *dnaAts* mutations. One of the most noticable side effects is the previously discussed cold sensitivity caused by *groE* over-expression in conjunction with certain *dnaAts* alleles (see section 4.4). Since this effect is allele-specific it is presumably directly related to the process of suppression. Additionally, the fact that all cold-sensitive alleles are also suppressible would tend to suggest that the processes are interconnected. It would seem likely then, that a detailed investigation into the the causes and consequences of cold-sensitivity should provide some insights into the mechanism by which suppression takes place.

The results presented in this chapter are perhaps somewhat different to the phenomena of cold-sensitivity, in that they may not be related to the process of suppression at all, either directly or indirectly. Indeed, they may merely represent the phenotypic consequences of the large scale production of a single protein species on the host cell physiology. However, they are included here for the sake of completeness, since it is not possible at this stage in time to determine whether these observations provide a real clue towards determining the function of the GroE proteins in the cell, and particularly their role in suppression, or whether they merely represent unconnected and irrelevant side effects. A case in point is the effect of nalidixic acid on strains overproducing GroE; does this demonstrate a role for GroE in the process of DNA replication, or is it merely the result of nalidixic acid causing an increase in expression of the *groE* genes and hence 'GroE poisoning'?

Certainly, the plethora of side effects caused by the over-expression of *groE*, whether relevant to the process of suppression or not, would tend to suggest a multitude of possible cellular interactions for these proteins. Even if the study of such effects may not provide many clues towards answering the question of how suppression might occur, it may provide valuable information regarding the normal intracellular functions of the *groE* gene products.

6.2 General effect of *groE* over-expression on cell growth

Over-expression of the *groE* genes can result in a general reduction in cellular growth rate in many strains. The effect of this seems to vary from strain to strain, and generally can be seen as a slightly reduced growth rate immediately following transformation with the *groE* plasmid pND5. In contrast to cold-sensitivity, this effect

is more noticable as the growth temperature is increased, suggesting that it is a consequence of the amount of GroE protein being made, since expression of the *groE* genes is temperature dependent (Herendeen *et al.* 1979). The other noticable aspect of the reduced growth rate of pND5-containing strains is that strains appear to become 'adapted' very quickly, and soon display a normal growth rate. An obvious explanation for this is that over-expression of the *groE* genes in these strains is being selected against, and mutations which either reduce plasmid copy number or reduce *groE* expression are occurring. Alternatively, plasmid located deletions may be taking place. This is superficially similar to the selection system against *groE* over-expression which gave rise to the plasmid copy number mutants described in chapter 7; however, the general reduction in growth rate caused by pND5 in most strains is negligable in comparison to the severe effect described as 'cold-sensitivity', and seen in conjunction with some *dnaAts* alleles.

TABLE 6.1 Effect of pND5 on growth rate in strain MM18

STRAIN	PLASMID	30°C	37°C	42°C
DOUBLING TIME IN MINUTES				
MM18	pND5	46	29	25
	pBR325	47	28	25

Unfortunately, this 'growth-rate reduction' is difficult to catagorise experimentally, since data often appears to be rather contradictory, possibly reflecting strain-dependent differences. For instance, MM18 or MM17 (MM18*recA*) were often transformed with pND5 as controls. As mentioned above, the growth rates of these cultures were often reduced, but these reduction was usually transient. Sometimes when MM18 was transformed with pND5 no difference could be seen. A comparison of the growth rates of MM18 pBR325 and MM18 pND5 at 30°C, 37°C and 42°C is shown in table 6.1. These represented newly transformed colonies which had been grown up in broth cultures overnight at 37°C prior to growth curves the next day. The presence of pND5 does not appear to affect the growth rate in MM18 (the isogenic *dnaA*$^+$ parental of the MM181-MM190 series) at all. However, at a later date, the plasmid contained in an MM18 pND5 culture was examined and found to comprise

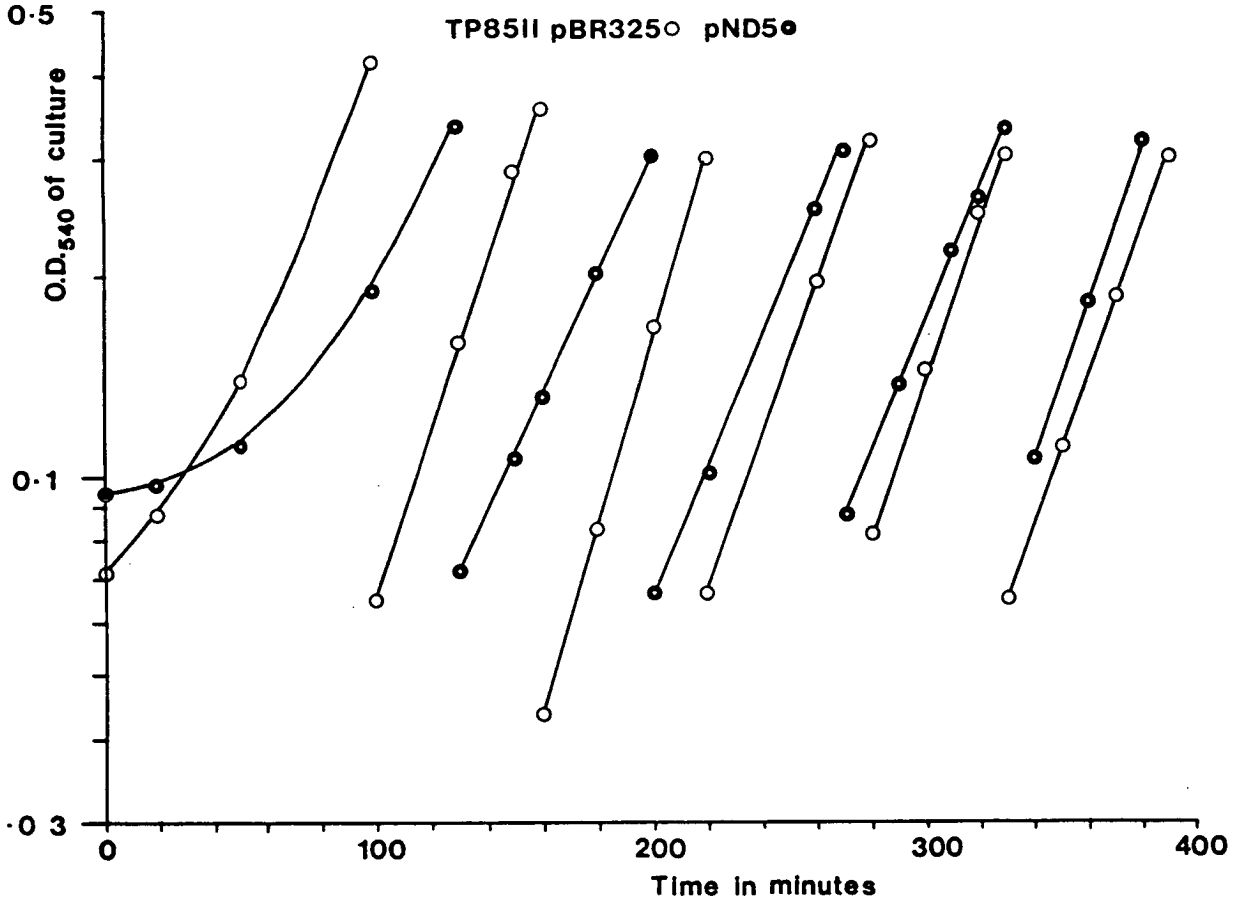
several species, including deletion derivatives and an intact pND5 (as shown by its ability to transform a *dnaAts* strain to temperature resistance). This tended to suggest that an intact pND5 probably is being selected against in MM18, although this is probably not a strong selective force.

When transformed into MM17, pND5 appears to be essentially stable, and no deletion derivatives were observed. Additionally, good expression of the plasmid-located *groE* genes had been observed (Jenkins *et al.* 1986). Was this merely due to the inability of this strain to excise the plasmid located *groE* genes, or did the mutant *recA* allele remove any deleterious effect of over-expression of *groE*? The growth rates in MM17 pND5 and MM17 pBR325 were examined and demonstrated that pND5 does appear to cause a reduction in growth rate in MM17, in contrast to the result seen with MM18, indicating that pND5 does cause a deleterious effect on growth in a *recA* background (data not shown). However, it is difficult to assess the real effect of pND5, since a number of possible explanations exist. pND5 may only affect MM17 *because* it carries a *recA* mutation, or alternatively, MM18 might be affected by pND5 as well, but deletion derivatives take over the population and mask this effect. As has been previously mentioned, the inconsistencies seen with regard to a growth-rate effect of pND5 make interpretation difficult. The growth rate of some strains appears to be noticably affected by pND5, while in other strains no effect can be seen. For example, pND5 was originally isolated in c600 (Jenkins 1985), in which it seemed to cause quite a severe reduction in growth rate. However, when transformed into the $dnaA^{+}recA^{+}$ strain TP91 no significant reduction in growth rate was observed. A growth curve showing TP91 pND5 compared to TP91 pBR325 is shown in figure 6.1. It is interesting to note that although the doubling time of both cultures eventually becomes essentially the same, the pND5-containing strain takes much longer to begin growing. Perhaps this is the cause of the initial 'slow-growth' phenotype noted for MM18 pND5 transformants. However, in another strain (ED2433) which was used at an early date during mapping experiments of the *sdaA* locus, pND5 caused a marked reduction in growth rate beyond the initial stages of the growth cycle, and additionally, this reduction worsened as the growth temperature was increased, as though the extent of the reduction were directly proportional to the amount of GroE over-production (data not shown).

It is perhaps easy to understand why pND5 might have a deleterious affect on cellular growth rate. Examination of the cellular protein content of a pND5 containing strain (see figure 3.7) reveals a vast over-expression compared to normal, with GroEL particularly making up a disproportionate amount of the total cellular protein. Even if

the resulting quantities of protein did not specifically affect certain metabolic processes, the strain upon the cells' metabolism in producing such amounts would be expected to be manifested as a reduction in growth rate.

FIGURE 6.1 Growth rates of TP91 pND5 and TP91 pBR325



Growth from stationary phase. Cultures diluted periodically to maintain OD between 0.05–0.2.

6.3 Effect of pND5 on host-cell resistance to nalidixic acid

6.3.1 Introduction

An effect upon the resistance to the antibiotic nalidixic acid in strains carrying the *groE* plasmid pND5 was first noted during experiments to map the *sdaA* locus by

Jenkins (1985). It was observed that when MM7 pND5 was grown on L-agar plates containing nalidixic acid at 20µg ml^{-1} a much reduced colony size was observed when compared to MM7 pBR325 controls. This is perhaps significant with regard to the mechanism by which *groE* is able to suppress *dnaAts* mutations, since the action of nalidixic acid is to inhibit DNA gyrase activity, thus causing a block to DNA replication (for review see Drlica 1984). DNA gyrase is essential for the elongation phase of DNA replication. Significantly, DNA gyrase also plays a crucial role in the initiation of DNA replication from *oriC* (for review see McMacken *et al.* 1987), and this protein has been shown to preferentially bind to a sequence near to the 245bp minimal origin region (Lother *et al.* 1984). It is thus eminently reasonable that the effect of *groE* over-expression on the resistance to nalidixic acid may be the result of an interaction between GroE and DNA gyrase during events occurring at the initiation of DNA replication. For this reason particularly it was considered of interest to investigate this effect.

The strains examined in this study are resistant to nalidixic acid by virtue of a *gyrA* mutant allele which they carry (which codes for the gyrase A subunit of the intact enzyme; Gellert *et al.* 1977). Thus the effect of pND5 upon the nalidixic acid resistance of an already resistant strain is being examined rather than its effect upon a wild type strain.

6.3.2 The *recA* mutation reduces nalidixic acid resistance in a *gyrA* strain

The original effect of ~~of~~ pND5 upon nalidixic acid resistance was first observed by Jenkins (1985) in the strain MM7 (*gyrA*). It was decided to further investigate the effect in this strain, and additionally in a *recA* derivative of MM7 called MM9, should deletion derivatives of pND5 become a significant problem. However, it was first decided to compare the nalidixic acid resistances of MM7 and MM9 in order to determine what effect the *recA* mutation had on the level of resistance. The strains were streaked out on L-agar plates and incubated overnight at 37°C. A fresh single colony was streaked out from this plate the next day onto L-agar containing various concentrations of nalidixic acid and incubated overnight at 37°C and the colony size compared to growth on L-agar alone was noted. These results are shown in table 6.2. It would appear that the *recA* mutation in MM9 has resulted in a considerable decrease in both the viability and growth rate of this strain when grown on higher concentrations of nalidixic acid. This is perhaps a not unexpected result, since the action of nalidixic acid is to inhibit gyrase action, and therefore would presumably introduce single-strand breaks into the DNA. This is essentially the same effect as

occurs following UV irradiation of a culture, and presumably the *recA* phenotype of MM9 results in a decreased efficiency of repairing such nicks. This would suggest that the resistance to nalidixic acid engendered by the *gyrA* allele is only partial in nature, and that a significant proportion is due to host-cell repair pathways.

TABLE 6.2 Nalidixic acid resistance of MM7 and MM9

STRAIN	CONCENTRATION OF NALIDIXIC ACID (ugs ml^{-1})				
	0	10	20	40	100
COLONY DIAMETER					
MM7 *recA*$^+$	1.0	1.0	1.2	1.2–1.5	1.5
MM9 *recA56*	1.0	1.0	1.0	0.6	-

Strains streaked to single colonies on L-Agar (+ nal) and incubated overnight (c.18hrs) whereupon colony diameter was measured.

6.3.3 A comparison of the effect of pND5 and growth temperature upon the nalidixic acid resistance in MM7 and MM9

Is the effect of pND5 upon nalidixic acid resistance due to the over-expression of the *groE* genes, or might it be due to over-expression of one of the other polypeptides encoded on the 8.1kb insert fragment? If this effect is indeed due to the over-expression of the *groE* genes, then it should be exacerbated as the temperature, and hence the level of *groE* expression is increased (Herendeen *et al.* 1979). It was therefore decided to compare the growth and viability of MM7 and MM9 containing either pND5 or pBR325 (as a control) on various levels of nalidixic acid and at various incubation temperatures, to see if the deleterious effect of pND5 is indeed temperature-influenced (as expected if over-expression of the *groE* genes is responsible).

Each culture was streaked out onto L-agar plates containing tetracycline at 5µg ml^{-1} to maintain selection for the plasmid. These were incubated overnight at 37°C, and a single colony of each used to innoculate plates containing various concentrations of nalidixic acid. These were then incubated overnight at either 30°C, 37°C or 42°C. Tetracycline was included as before. The results of this experiment, presented as the viability compared to an L-agar only plate, are shown in table 6.3.

TABLE 6.3 Effect of pND5 on nalidixic acid resistance of MM7 and MM9

STRAIN	PLASMID	CONCENTRATION OF NALIDIXIC ACID (ug ml^{-1}) 0	10	20	40	80
VIABILITY						
MM7	pBR325 30°C	+++	+++	+++	+++	++
recA$^+$	pBR325 37°C	+++	+++	+++	+++	++
	pBR325 42°C	+++	+++	+++	++	+
	pND5 30°C	+++	+++	++	+	–
	pND5 37°C	+++	+++	++	+/–	–
	pND5 42°C	+++	++	+	+/–	–
MM9	pBR325 30°C	+++	++	+	–	–
recA56	pBR325 37°C	+++	++	+	–	–
	pBR325 42°C	+++	++	+	–	–
	pND5 30°C	+++	+++	+	–	–
	pND5 37°C	+++	+++	+	–	–
	pND5 42°C	+++	+++	+	–	–

Cultures were patched on LA plates (+ or - nalidixic acid) and incubated overnight (c. 18hrs) whereupon a subjective estimate of viability was made.

As already observed, MM9 generally exhibits a lower resistance to nalidixic acid than MM7. However, temperature and the presence of pND5 appear to have no affect upon the resistance level in this strain. In fact, colony size appears to be slightly larger as the temperature is increased for the higher concentrations of nalidixic acid, although this may simply be due to the breakdown of the antibiotic at the higher temperatures rather than any specific effect. In contrast, with MM7, the presence of pND5 and the effect of temperature changes seem to have a very marked effect on viability and growth rate. Although the presence of pND5 appears to cause a slight reduction in the growth rate of this strain under all conditions, this effect is multiplied as the nalidixic acid concentration is increased. Additionally, this reduction in growth

rate is much worse as the temperature is increased, as would be expected if the deleterious effect of pND5 resulted from over-expression of *groE*.

Thus it would seem likely that over-expression of the *groE* genes is indeed responsible for the reduction in resistance to nalidixic acid observed in strains carrying pND5. Why is it that this effect is not seen in MM9 though? Is it possible that sufficient over-expression of the *groE* genes does not occur in a *recA* strain, even though the genes are cloned in high copy number? This is probably not the case, since suppression of *dnaAts* mutations by pND5 in a *recA* background can occur, indicating that over-expression must be taking place. In addition, examination of GroE protein levels in a *recA* strain has demonstrated as much over-expression as observed in a *rec*$^+$ background (Jenkins *et al.* 1986 and figure 3.7). The most likely explanation is that resistance levels in MM9 are so low anyway (due to the presence of the *recA* mutation) that the extra effect of pND5 is negligible and liable to be masked. Even with pND5, MM7 is still able to grow on nalidixic acid plates at a concentration of 100µg ml^{-1}, although the growth rate is severely reduced. In contrast, MM9 is unable to grow above 40µg ml^{-1} under any conditions.

6.3.4 The growth rate in liquid media of MM7 pND5 and MM7 pBR325

In order to gain a more quantitative estimate of the effect of pND5 on the resistance of the host strain to nalidixic acid, it was decided to measure the growth rates of liquid cultures in various concentrations of nalidixic acid and at various growth temperatures. This was performed for MM7 pND5 and MM7 pBR325 only, since the resistance levels of MM9 appeared to be essentially unaffected by the presence of pND5.

Fresh overnight cultures which had been grown to stationary phase in L-broth containing tetracycline at 5µg ml^{-1} at a growth temperature of 37°C were diluted down the next day into pre-warmed broth and the growth followed (i.e. the O.D.$_{540}$). The growth media contained tetracycline to maintain selection for the plasmid, and nalidixic acid at either 0, 10, 40 or 100µg ml^{-1}. The cultures were grown at either 37°C or 42°C for a period of several hours. The resulting growth curves are shown in figure 6.2 (MM7 pND5) and figure 6.3 (MM7 pBR325). The presence of pND5 results in amplification of the effect of nalidixic acid on the host strain, this effect being worse as the temperature is increased. This temperature effect is even seen with the MM7 pBR325 control, suggesting that over-expression of the *groE* genes is occurring even in the absence of pND5 to a level which is deleterious in conjunction with nalidixic

FIGURE 6.2 Growth curves of MM7 pND5 at different temperatures and different nalidixic acid concentrations

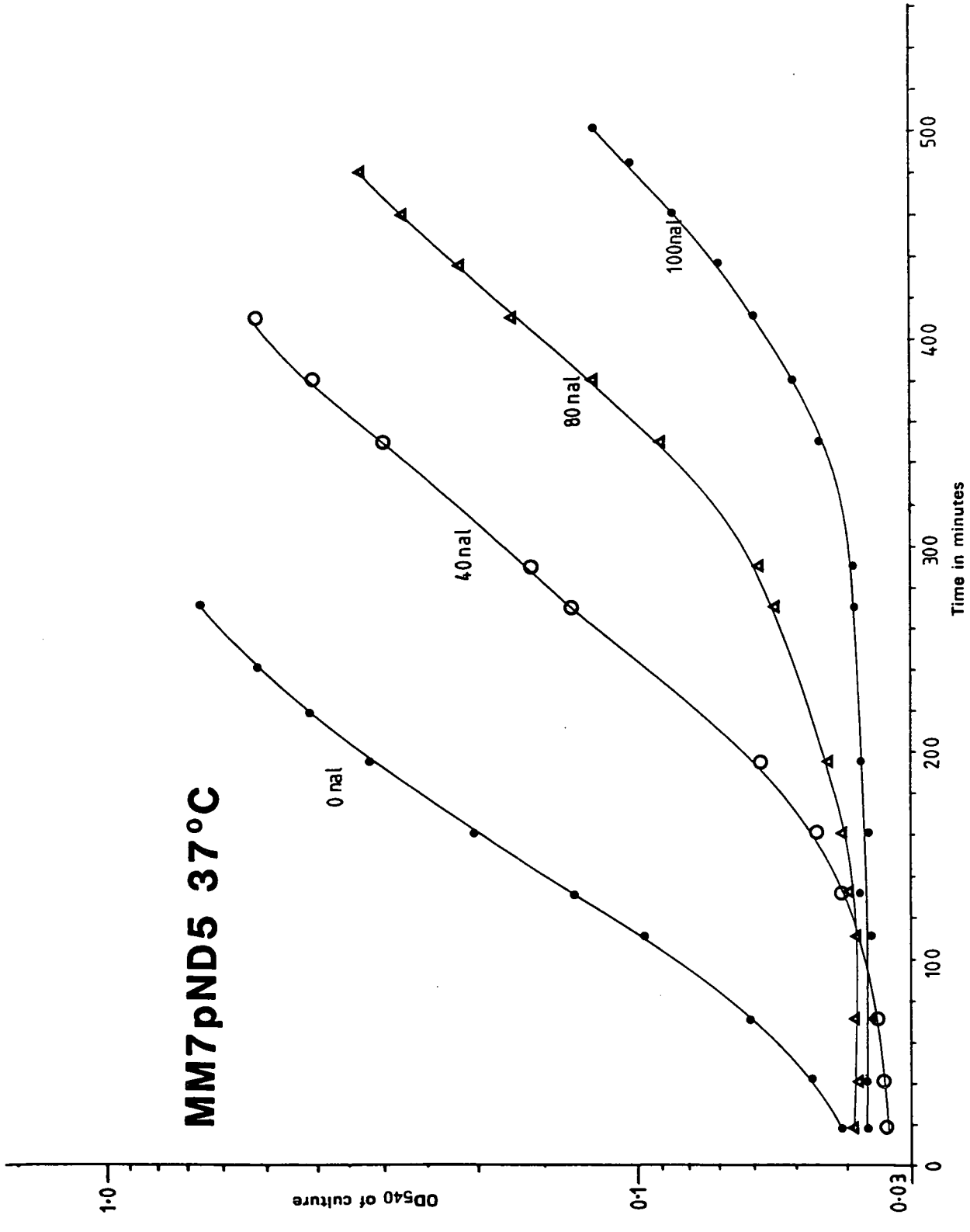


Figure 6.2: (continued)

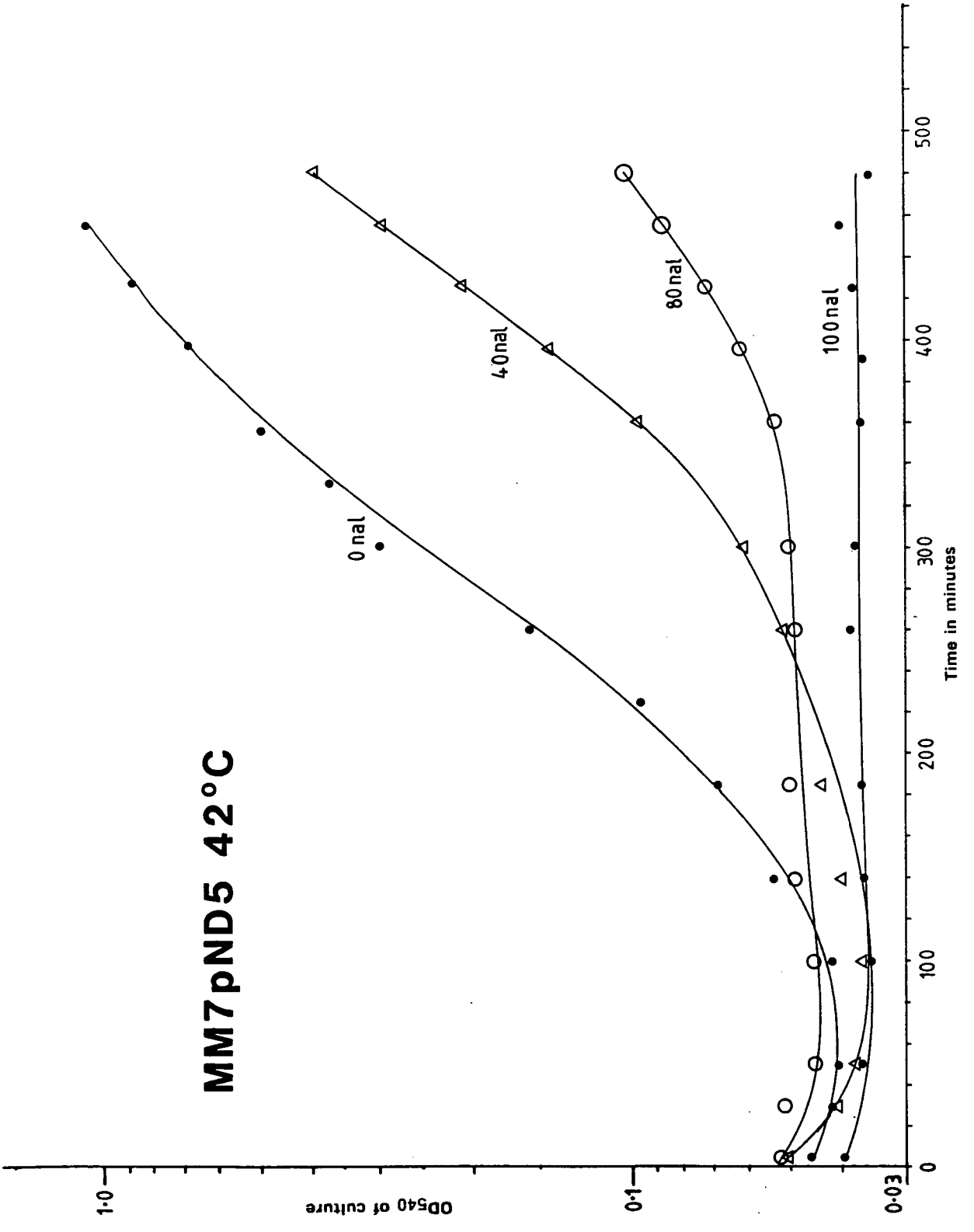


FIGURE 6.3 Growth curves of MM7 pBR325 at different temperatures and different nalidixic acid concentrations

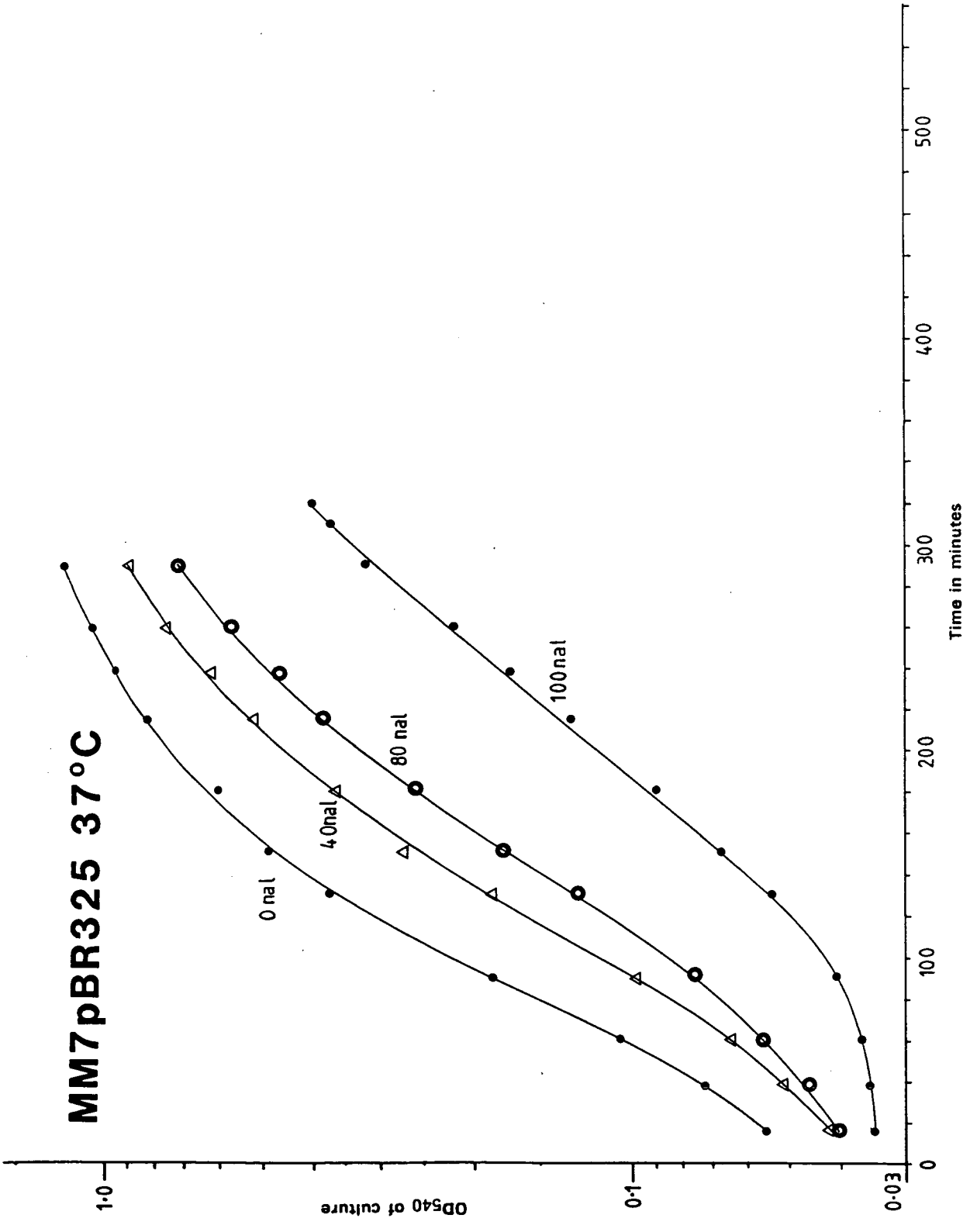
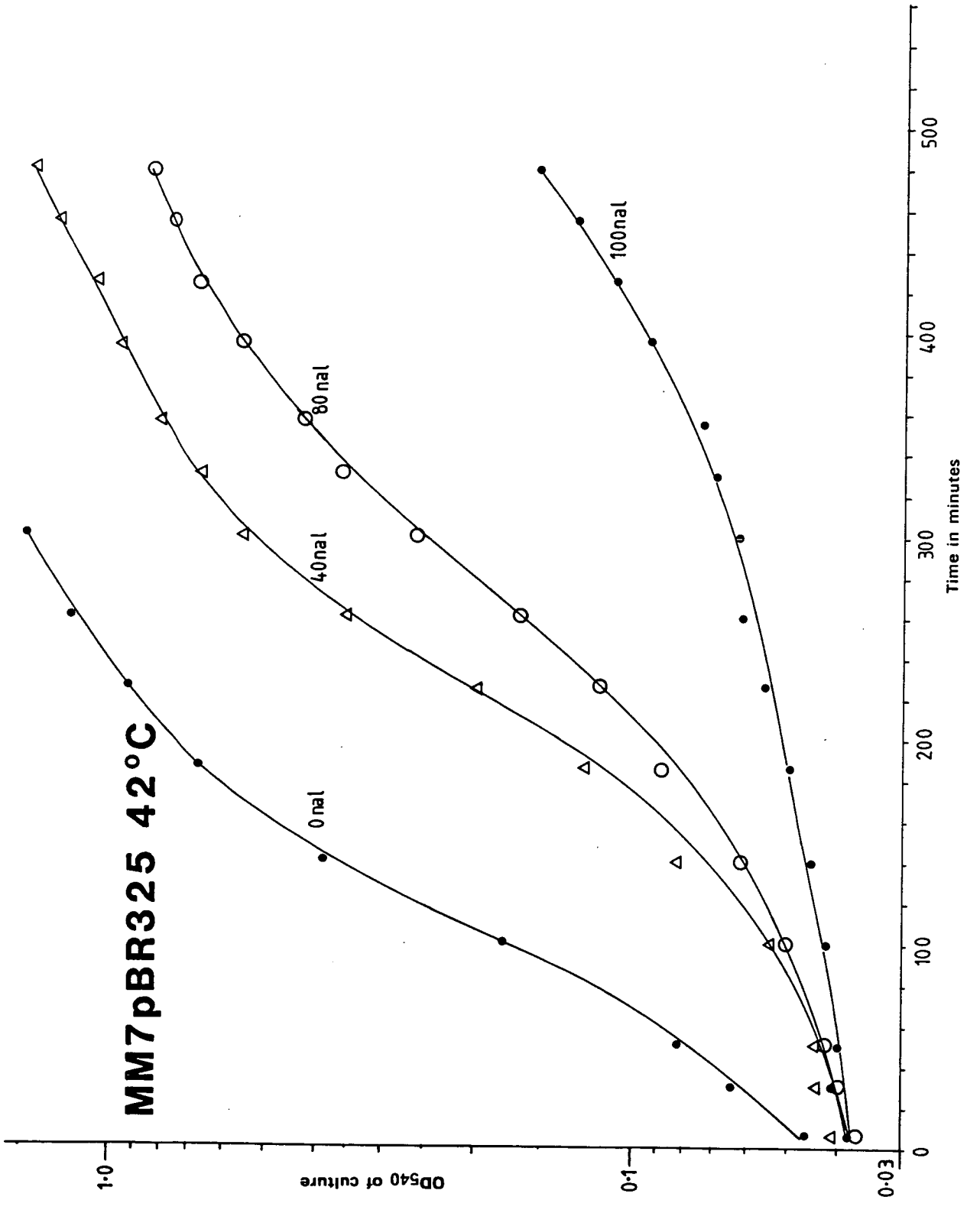


Figure 6.3: (continued)



acid. What is most interesting perhaps is the manner in which nalidixic acid and pND5/temperature combine to effect the growth rate. Rather than reducing the growth rate *per se*, they appear to introduce a lag period, after which cells begin to grow again at a nearly normal rate. The period of lag seems to be both temperature and pND5-dependent; a much longer lag is apparent at 42°C than 37°C, and increased still further when pND5 is present. This would seem to suggest that the cells undergo a period of 'adaption' following challenge with nalidixic acid before they are able to start growing again, and the length of this lag appears to be influenced by factors which affect the GroE protein levels (i.e. temperature and the presence of the *groE* genes on a high copy plasmid).

6.3.5 Effect of pre-growth in nalidixic acid

If this 'adaption' hypothesis is correct, then cultures pre-grown in nalidixic acid should not exhibit this temporary cessation in growth when diluted back into fresh nalidixic acid-containing media, while those pre-grown in L-broth alone would be expected to do so. To test this, overnights of MM7 pND5 and MM7 pBR325 were grown to stationary phase in L-broth containing either tetracycline at 5μg ml^{-1}, or tetracycline at 5μg ml^{-1} and nalidixic acid at 40μg ml^{-1}, at 37°C. These were then diluted back the following day and grown under the same conditions until steady-state growth had been achieved. The cultures being grown in L-broth containing tetracycline alone were then diluted back into the same media but supplemented with nalidixic acid at 40μg ml^{-1}. The growth curves obtained from this experiment are shown in figure 6.4. It is evident that cultures pre-grown in media containing nalidixic acid show no lag when diluted back into fresh nalidixic acid supplemented media, whether growth is in mid-log phase or from stationary phase. However, a significant lag period is seen with cultures that have not been pre-grown in nalidixic acid, whether in mid-log phase growth (as was examined in this experiment; figure 6.4) or diluted back from a stationary phase culture (as has already been seen in figures 6.2 and 6.3). It is also apparent that the reduction in growth caused by nalidixic acid is far more severe when pND5 is present in the strain.

6.3.6 Discussion

Why should pND5 have this effect on the nalidixic acid resistance of the host strain, and what is the cause of this lag period? One possibility is that this effect is due to an interaction between GroE and gyrase during the replication of DNA. If the proteins interact in some unspecified manner, it could be that the presence of extra GroE protein somehow makes gyrase more susceptible to the action of nalidixic acid.

Presumably some sort of adaptive process is occurring in the cell during the observed lag period following challenge with nalidixic acid. An alternative hypothesis could be that the effect of nalidixic acid is merely to cause an increase in expression of the *groE* genes; the level of this induction being magnified as the temperature is increased. This hypothesis would suggest that it is simply the large quantities of GroE

FIGURE 6.4 Growth curves of MM7 pND5 and MM7 pBR325 following pre-growth in nalidixic acid containing medium

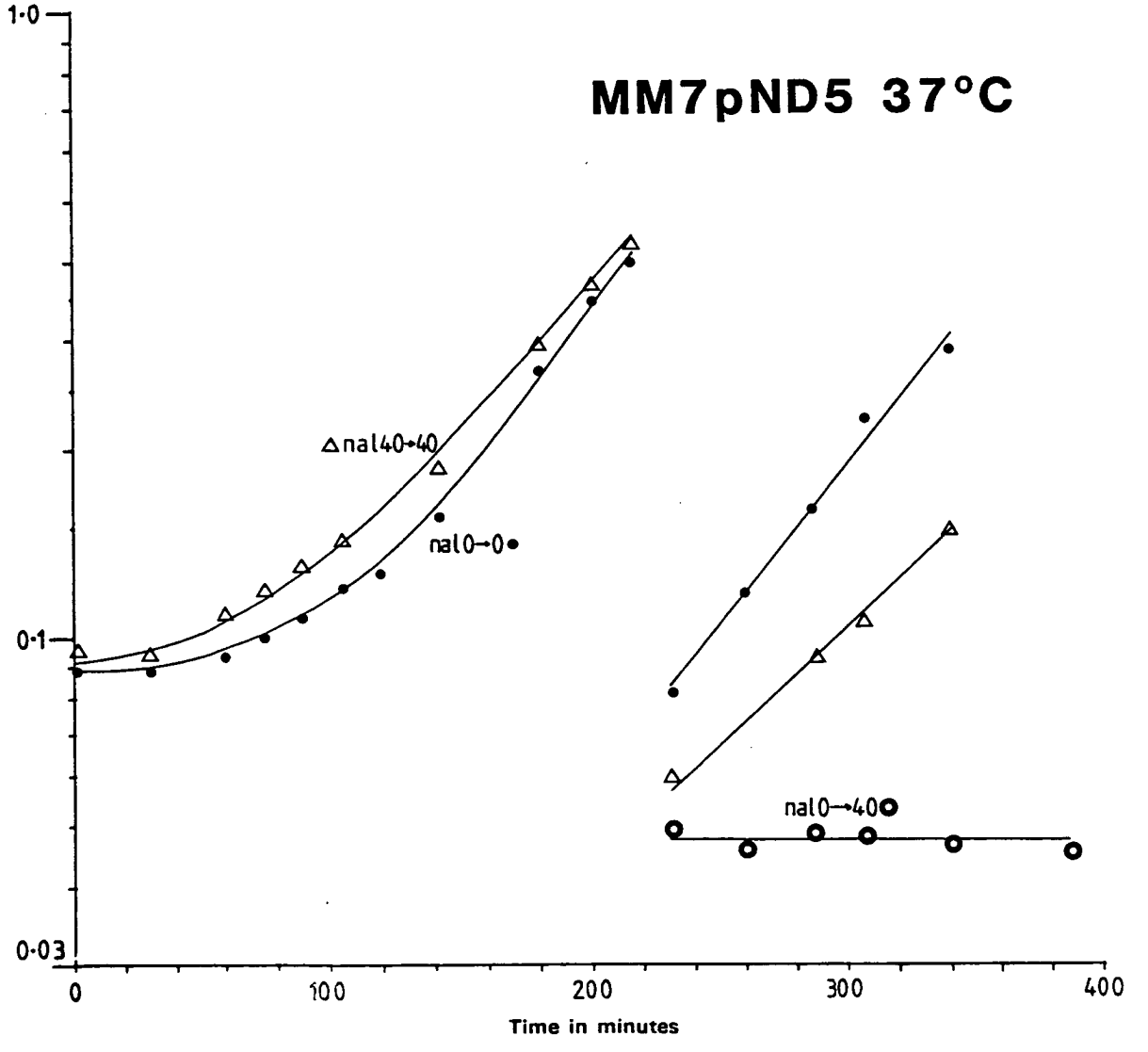
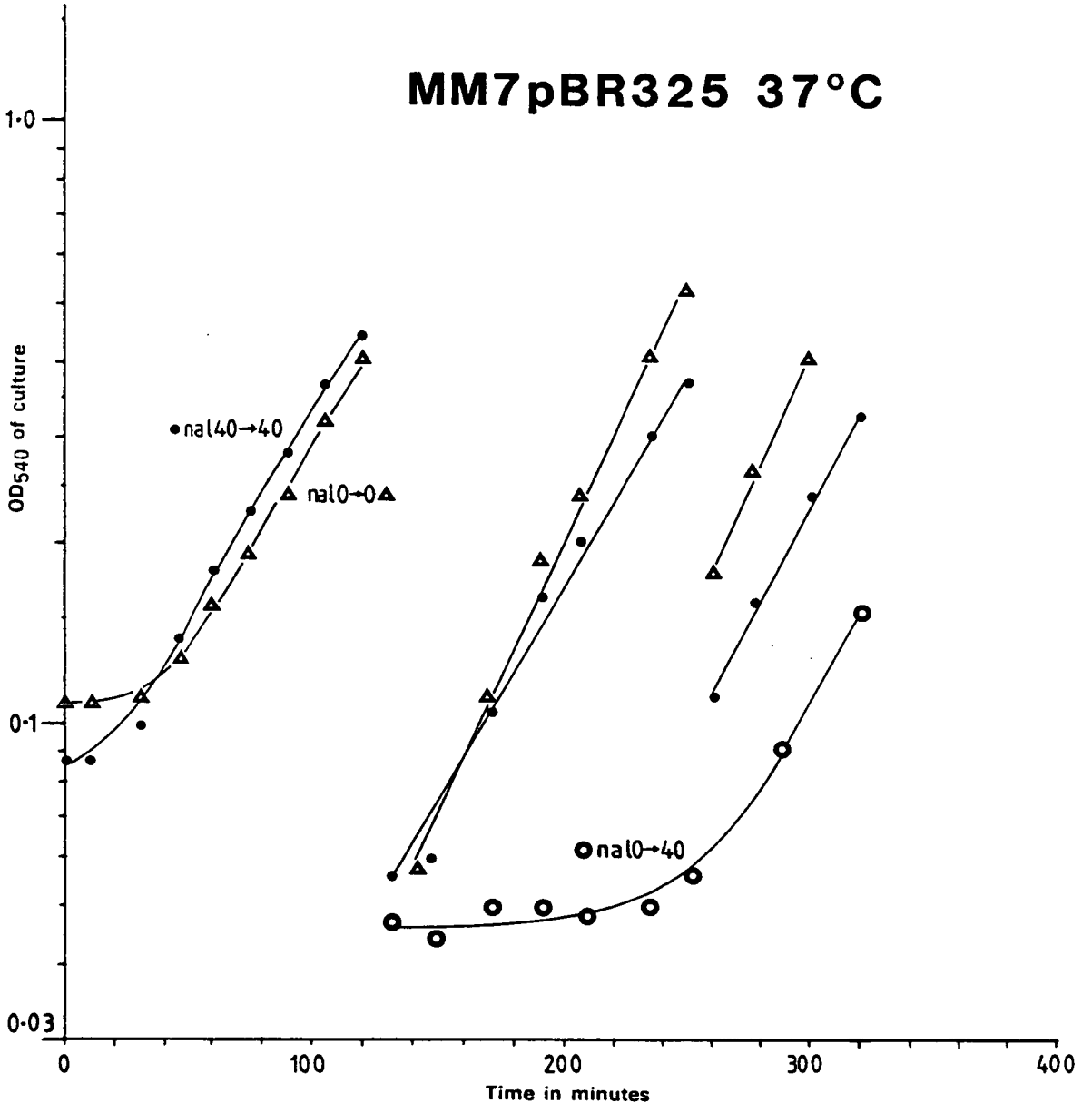


Figure 6.4: (continued)



protein present under such conditions which is the cause of the observed effect (i.e. GroE poisoning). It is certainly known that *groE* expression is induced by nalidixic acid (Krueger and Walker 1984, VanBogelen *et al.* 1987), and it is possible that the observed lag period is simply due to the transient increase in expression while the cells are in a period of 'heat shock' (i.e. challenge with nalidixic acid). However, the cultures are being grown continuously in media containing nalidixic acid; the induction of *groE* expression (if this is the cause of the reduced growth) should be continous and not

transient as suggested by the length of the lag period. In addition, this lag is also seen in control strains lacking pND5, in which the level of *groE* expression would be expected to be much less pronounced. Thus it would seem more likely that over-production of GroE affects nalidixic acid resistance in some specific fashion rather than simply by virtue of the fact that large quantities of an unnecessary protein are placing too great a strain upon the cells' metabolism.

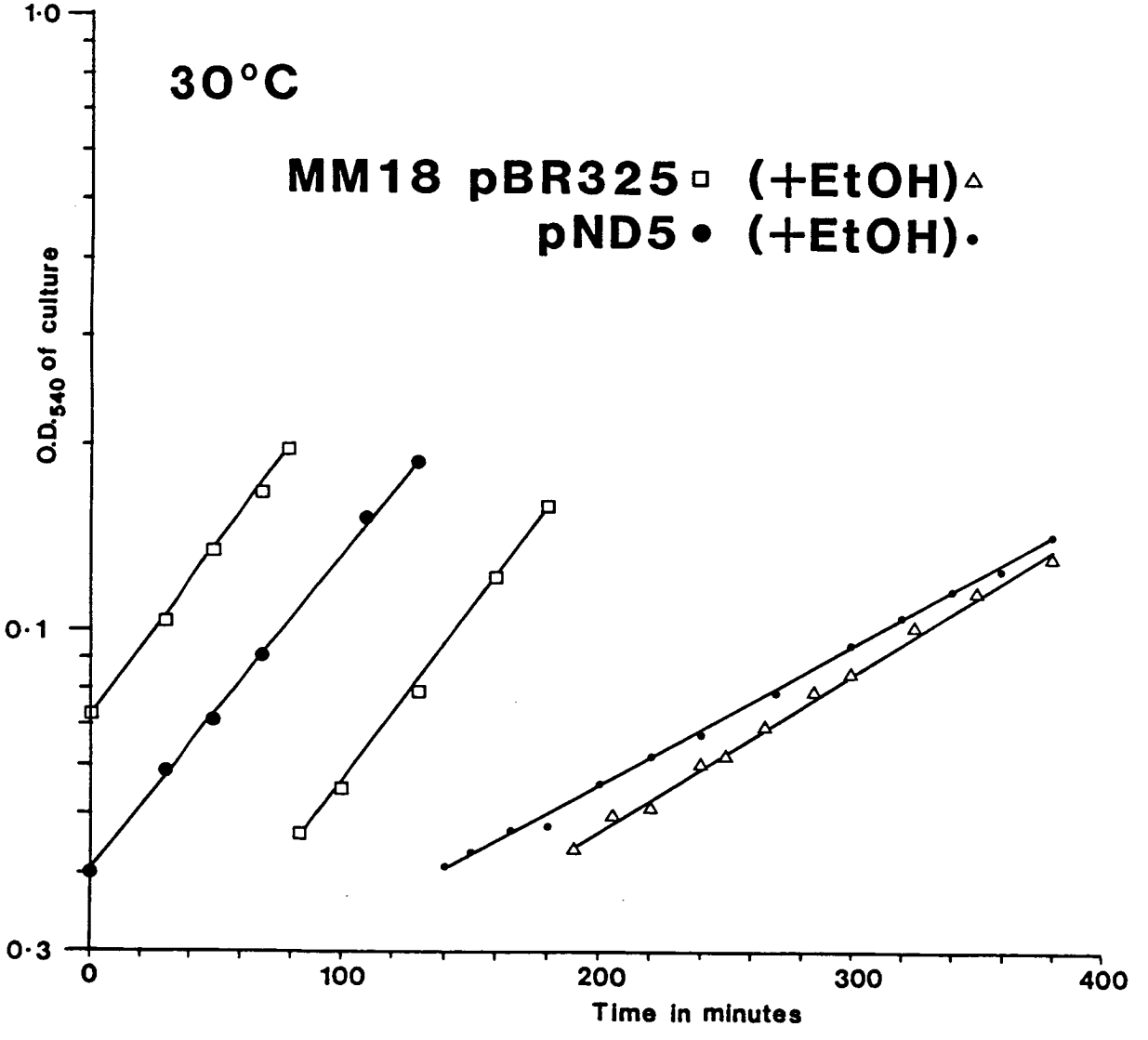
6.4 Effect of pND5 on ethanol shock and UV resistance

It has already been suggested that GroE may interact with gyrase and thus affect its susceptibility to nalidixic acid poisoning; another possibility is that excessive quantities of GroE may affect the host cells' ability to turn on the heat shock response if it perhaps can act in some sort of regulatory fashion (section 4.6.2). This has already been discussed as a possible mechanism by which GroE may suppress *dnaAts* mutations; the prevention of the induction of proteases to degrade the temperature sensitive protein. It was additionally noted that cultures containing pND5 showed a reduced survival at higher temperatures, as would perhaps be expected if induction of the heat shock response were being prevented, although it was concluded that this effect could simply be due to the over-production of GroE straining the cells' metabolism (i.e. GroE poisoning). The effect of nalidixic acid is similar, in that differentiation between a specific gyrase-GroE interaction, and simple over-expression is difficult to make. However, another agent which induces the heat shock response includes ethanol, which results in mistranslation (So and Davie 1964), a disruption of transmembrane transport (Ingram and Buttke 1984), and accumulation of intracellular ppGpp levels (Lee *et al.* 1983). Thus, although the end result is the same (i.e. induction of the heat shock response), the events leading to induction are different, and induction by ethanol should not affect gyrase action and introduce single stranded nicks as does nalidixic acid. Thus if ethanol shock in the presence of pND5 also results in a reduced viability, it would seem likely that the effect seen with nalidixic acid is simply due to the large amounts of GroE protein made during these conditions. If however, a reduced viability/growth rate is not seen in conjunction with pND5, then this would suggest that the reduced viability seen with nalidixic acid is specific to an effect between GroE, gyrase and nalidixic acid, or an additional effect not related to heatshock induction. With this in mind it was decided to examine the effect of pND5 upon a host strain subjected to ethanol shock. Additionally, the effect of UV irradiation on a pND5-containing strain was examined also.

6.4.1 Growth curves and viability following ethanol shock

Cultures of MM18 pND5 and MM18 pBR325 which had been freshly transformed and checked for the presence of the intact plasmids were grown overnight in L-broth containing tetracycline at 5μg ml^{-1}. Next day these cultures were diluted back and grown under the same conditions for several hours, diluting back as required and maintaining the $O.D._{540}$ between 0.04 and 0.2. After steady-state growth

FIGURE 6.5 Growth curves of MM185 pND5 and MM185 pBR325 following ethanol shock



had been achieved, the culture was subjected to ethanol shock by diluting back 5mls of culture into 15ml of L-broth + ethanol (14.2ml L-broth + 0.8ml ethanol). This procedure resulted in a final ethanol concentration of 4%, while avoiding the risk of high localised ethanol concentrations. Cell size distributions were monitored throughout the experiment also. The growth curves obtained in this experiment are shown in figure 6.5. No significant difference in response is seen, with both cultures exhibiting a marked reduction in growth rate following the addition of ethanol, although that of the pND5-containing culture does seem slightly more reduced after ethanol shock, although prior to ethanol shock a slightly reduced growth rate is apparent also. Examination of the cell size distributions (data not shown) showed no significant difference either, with both cultures exhibiting a 5% reduction in cell size over a 120 minute period.

Thus the response of the host strain to ethanol shock appears to be unaffected by the presence of pND5. The viability of the culture was examined by diluting a stationary phase culture (1:100) of either MM18 pND5 or MM18 pBR325 into L-broth containing various concentrations of ethanol (0-10%), leaving at 30°C for two hours, and then diluting down to single colonies on L-agar plates using fresh pre-warmed broth. These were incubated overnight at 30°C and the number of colonies on each plate was then counted. The results are shown in table 6.4, and demonstrate no significant difference in resistance to ethanol shock caused by the presence of pND5. Additionally, they would suggest that 3-4% ethanol in the growth medium is the maximum amount before a significant drop in viability is seen.

TABLE 6.4 Effect of pND5 upon cell viability following challenge with ethanol

STRAIN	ETHANOL CONCENTRATION (%)					
	0	1	2	3	4	5
NO OF COLONIES						
MM18 pND5	1726	1757	1734	1186	434	6
MM18 pBR325	1763	1636	1563	1062	242	48

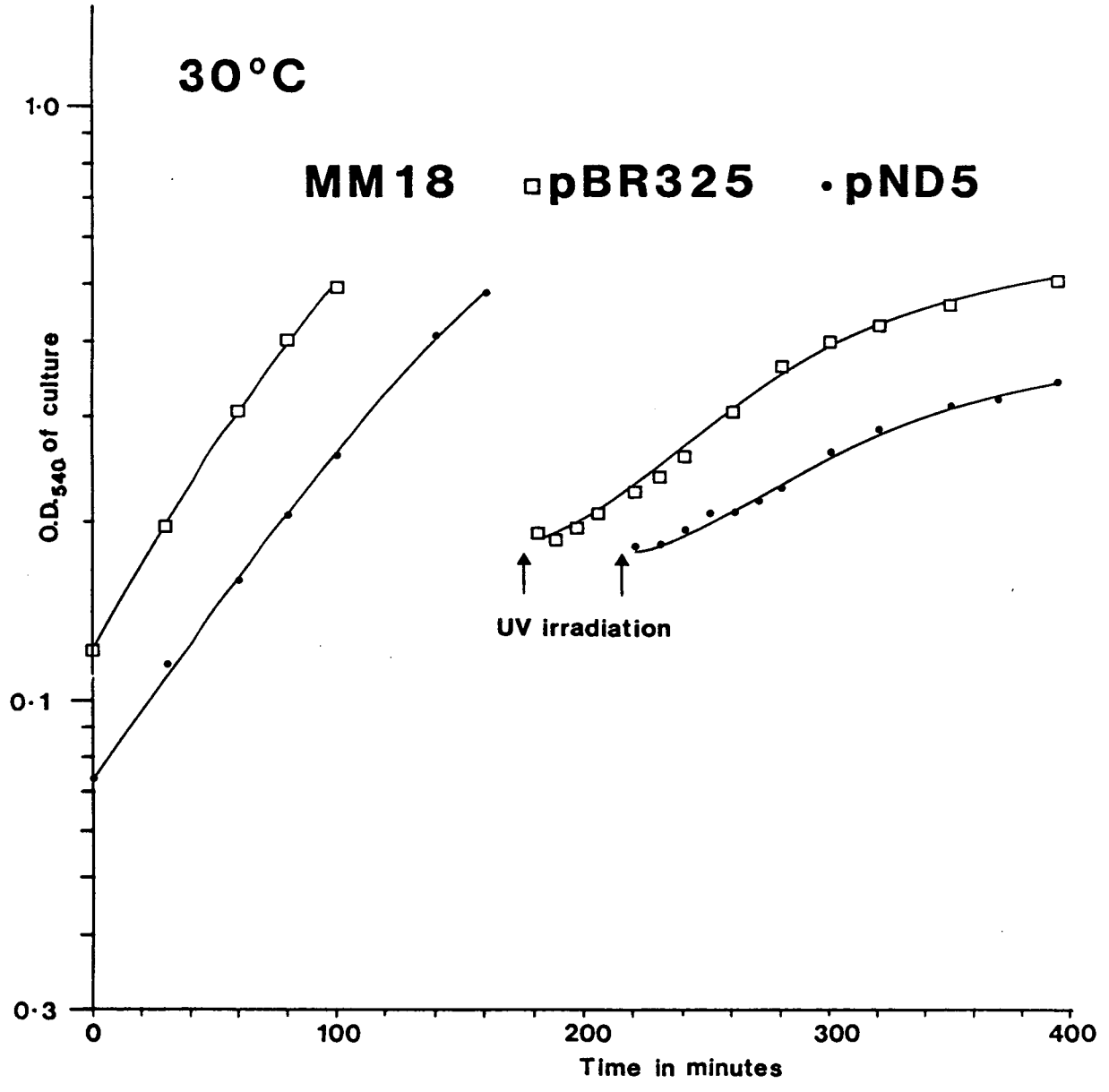
See text for details.

These results would suggest that the reduction in viability and growth rate caused by nalidixic acid in conjunction with pND5 is a specific effect, possibly due to an interaction between GroE and Gyrase, rather than a simple case of GroE poisoning. Ethanol shock does not result in the long growth lag period observed with nalidixic acid. It is unlikely therefore that this lag is due to the time taken to switch off the heat shock response following induction with nalidixic acid, and is more likely to be due to some kind of adaptive process. Additionally, ethanol shock does not result in a reduced viability as observed with nalidixic acid in conjunction with pND5. Since ethanol is in fact a more efficient inducer of the heat shock response than nalidixic acid (Neidhardt and VanBogelen 1987), if the observed reduction in viability following nalidixic acid with MM7 was simply due to GroE poisoning, then this effect should be even worse following ethanol shock. This is not the case, and so would suggest that the phenotypic effects seen following nalidixic acid shock in conjunction with pND5 (a reduced viability and a long period of adaption) are due to some specific interaction between GroE, gyrase and nalidixic acid.

6.4.2 Growth curves and viability following UV irradiation

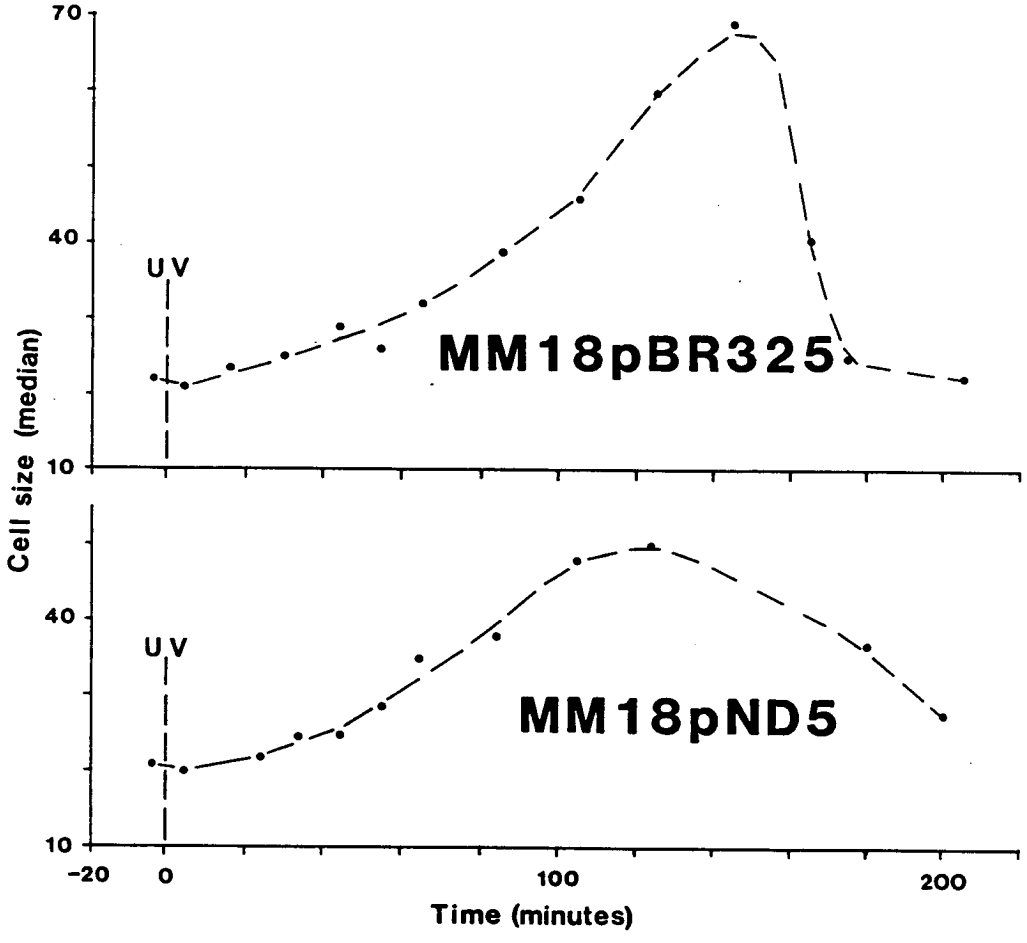
The effect of UV irradiation on strains MM18 pND5 and MM18 pBR325 was examined by growing both cultures to mid-log phase in L-broth with tetracycline selection at 37°C. 5ml of these cultures was then placed on ice for 10 minutes, pelletted in a bench top centrifuge (cold room), and resuspended in 5ml of ice-cold 10mM $MgSO_4$. This was transferred to a sterile glass petri dish and irradiated with 450ergs of ultraviolet light, prior to transfer to 15ml of fresh pre-warmed filtered L-broth. The increase in optical density and cell numbers was then monitored. The viability of each culture was estimated by taking an aliquot in $MgSO_4$ immediately prior to UV irradiation, and comparing the viable counts to an identical aliquot taken immediately following irradiation. The growth curves of the cultures ($O.D._{540}$) are shown in figure 6.6. Both cultures seem to behave similarly, exhibiting a lag following UV irradiation and a subsequently reduced growth rate. The cell numbers and size distributions are shown in figure 6.7. Both cultures show a period during which cell numbers fail to increase, and the cells consequently filament. The subsequent decrease in cell size is accompanied by an increase in cell numbers as the culture begins to divide again following the switch-off of the SOS-response. Thus both cultures appear to shown an essentially normal response (for a review of the physiological events following induction of the SOS-response see Walker 1987). Additionally, viable counts following UV irradiation failed to show any significant

FIGURE 6.6 Growth curves of MM185 pND5 and MM185 pBR325 following UV irradiation



difference between the cultures (data not shown). In common with ethanol shock then, UV irradiation does not cause a significantly greater effect when the host strain carries pND5.

FIGURE 6.7 Cell sizes of MM185 pND5 and MM185 pBR325 following ethanol shock



6.5 Summary of the effect of pND5 upon the *E.coli* stress response

It was noted in chapter 4 (section 4.6.2) that strains carrying pND5 appeared to be more sensitive to heat shock than controls, although such strains appeared to filament normally, suggesting that pND5 was not preventing induction of the heat shock response. A possible explanation of this effect is that greater expression of the *groE* genes is resulting in 'GroE poisoning' under such conditions. Plasmid pND5 also affects the nalidixic acid resistance of a *gyrA* mutant host strain. Transfer to a growth medium containing nalidixic acid results in a lag period before growth resumes; the

length of these lag, and the subsequent reduction in growth rate and viability is both temperature and pND5 dependent, suggesting that it is due to the amount of GroE protein present. This lag is not seen following ethanol shock or UV irradiation (inducers to the heat shock and SOS responses respectively), which also induce *groE* expression (ethanol to a greater and UV irradiation to a lesser extent; Krueger and Walker 1984). This suggests that the effects of pND5 in conjunction with nalidixic acid are not simply caused by 'GroE poisoning', but are due to a specific interaction between nalidixic acid, gyrase and GroE. A more definitive way of examining this would be to make use of the *groE* genes under the control of an inducible promoter such as the *lacUV5* promoter. Over-expression of *groE* could then be separated from induction of the rest of the heat shock response. The ideal situation would be to use a strain unable to induce heat shock (i.e. an *htpR* null mutant); however, the recent observation that such strains are inviable above 20°C effectively precludes this approach (Zhou *et al.* 1988). However, if the *groE* genes were contained on a high copy plasmid under control of the *lacUV5* promoter for example, addition of nalidixic acid should not result in any further expression of the *groE* genes; thus 'GroE poisoning' could be eliminated. Even without this, the available data would tend to suggest that the observed reduction in nalidixic acid resistance in a pND5-containing strain is probably due to some specific interaction, strengthening the case for GroE interacting during the process of DNA replication.

Why should over-expression of *groE* in conjunction with nalidixic acid cause such a long lag in the growth rate? Evidently a transient induction of the heat shock response (by the addition of nalidixic acid) cannot explain such a prolonged response. Here the realms of speculation must be entered, although an interesting model, and one which would additionally explain the absence of an effect of pND5 in a *recA* strain may be considered. Ultraviolet irradiation is a powerful inducer of the SOS response in *E.coli*, by virtue of the single stranded breaks present in the newly replicated DNA. It is a pulse inducer of SOS; a more prolonged inhibition of replication would result if the SOS response were continuously induced. If GroE were to interact with gyrase and thus reduce its resistance to nalidixic acid, single stranded breaks would be expected to occur, causing in a prolonged induction of the SOS response. This may be the cause of the extended lag period in pND5-containing cultures; induction of the SOS response rather than heat shock. Presumably the cell must eventually become adapted in some manner and thus allow growth to proceed. Such a model would explain why no additional effect upon nalidixic acid resistance was observed in a *recA* strain over-expressing *groE*; although the single stranded breaks would still be present, induction of SOS would not occur.

6.6 Effect of 8.1kb *groE* DNA fragment on the host cell resistance to ampicillin

From the very outset of work on the *sdaA* suppressor it appeared that the 8.1kb *groE* fragment caused an effect upon the host cell resistance to the antibiotic ampicillin. Strains containing the *groE* plasmid pND5 frequently appeared to form smaller colonies when grown on plates containing ampicillin as the selective agent, and the fact that this effect appeared more pronounced with an increase in temperature suggested the involvement of one or more of the *groE* gene products.

Ampicillin is one of the β-lactam class of antibiotics (penicillins), and acts to inhibit peptidoglycan biosynthesis; specifically the cross-linking of linear peptidoglycan chains. It thus affects the process of cell well synthesis, and at low concentrations preferentially inhibits septation, while higher concentrations lead to a general cell lysis (for reviews of the action of β-lactam antibiotics see Matsuhashi *et al.* 1982 and Waxman and Strominger 1983). The enzyme β-lactamase (product of the *amp* gene) confers host-cell resistance to ampicillin. This gene is present on the cloning vector pBR322 and most of its derivatives, including the *groE* plasmid pND5 under study here. β-lactamase is a periplasmic protein which is synthesised in a precursor form and is processed to the mature form during secretion into the periplasmic space (Josefsson and Randall 1981, Koshland and Botstein 1982, Muller and Blobel 1984). Since the precursor form of β-lactamse is enzymatically inactive (Roggenkamp *et al.* 1981), post-translational control of β-lactamase activity is highly probable, a topic I shall return to later with regard to the possible involvement of GroE in determining ampicillin resistance.

6.6.1 The effect of pND5 on ampicillin resistance

Initial observations with pND5 suggested that the *groE* genes in high copy were affecting ampicillin resistance, although as with the general effect of this plasmid on cellular growth rate, these results were highly inconsistent and strain-specific. When transformed into MM18, pND5 initially caused a slight reduction in colony size on ampicillin plates at 42°C compared to tetracycline controls. This effect was not seen with MM18 pBR325. However, the differential growth rates only persisted for a short time following transformation with pND5; once subcultured this effect was quickly lost. The effect appeared to be more severe for strain ED2433 when transformed with pND5; colonies were unable to grow on ampicillin plates at 42°C but could grow on tetracycline plates at this temperature. However, a generally more severe reduction in growth rate caused by pND5 has already been noted even in the absence of this ampicillin effect in this strain. These initial observations were not

pursued further since the most likely explanation seemed to be that strong read-through from the *groE* promoter was probably reducing transcription of the *amp* gene or possibly affecting the plasmid copy number. Such affects have been noted before (Steuber and Bujard 1982, Caulcott *et al.* 1985), so it seemed unlikely that any specific effect on ampicillin resistance by GroE was occurring.

The possibility that over-expression of *groE* might be directly affecting ampicillin resistance became more likely following results obtained with the low-copy *dnaAts* strain JM21 (see chapter 7). This strain carries the *pcnB* mutation, which lowers pBR-type copy number to about 20-30% of wild-type levels. An examination of GroE protein levels in JM21 pND5 compared to the isogenic parental MM185 pND5 had revealed considerably reduced quantities in the former, and together with additional data had suggested a reduced copy number for the plasmid in this strain (which was later confirmed following quantitative hybridisation measurements). An examination of the ampicillin resistance levels conferred by pND5 in each of these strains had shown identical levels of resistance though. Additionally, although the ampicillin resistance of pND5 in MM185 was only 800µg ml^{-1} compared to an ampicillin resistance level of 2000µg ml^{-1} for pBR325 in MM185, quantitative hybridisation data showed the plasmids to have an essentially identical copy number (chapter 8). What might be the cause of the reduced ampicillin resistance of pND5? A possible explanation is that read-through transcription is reducing the expression of the *amp* gene in the pND5 construct. However, if this is the case, then whatever level of ampicillin resistance is conferred in MM185 pND5 should be reduced to about 20-30% in JM21. This is not seen. Instead, the most likely explanation is that the amount of GroE directly affects the ampicillin resistance level. In MM185 pND5 the plasmid is present in a high copy number, so although the level of expression of the *amp* gene is high, so too is the amount of GroE. Conversely, in JM21 pND5 the *amp* gene dosage is lower, but so too is the amount of GroE protein to affect the ampicillin resistance.

6.6.2 The effect of λ*sidA* on ampicillin resistance

However, to prove that this is indeed a *trans* acting effect of GroE on the ampicillin resistance, and not due to some unforseen effect of the 8.1kb insert fragment on the expression of the *amp* gene, it was decided to see if the same effect could be caused by over-expression of the *groE* genes from λ*sdaA* in conjunction with a strain carrying pND5. The *dnaA46ts* strain MM185 (pBR325) was used to test

TABLE 6.5 Effect of λ *sidA* upon ampicillin resistance in MM185 pBR325

LYSOGEN TYPE[a]	TEMPERATURE[b]	AMPICILLIN CONCENTRATION (ug ml^{-1}) 0	50	400	800	1600	2000
VIABILITY							
λ616	30°C	+	+	+	+	+	+
(30°C)	37°C	–	–	–	–	–	–
	42°C	–	–	–	–	–	–
λ *sidA*	30°C	+	+	+	+	+	+
(30°C)	37°C	–	–	–	–	–	–
	42°C	–	–	–	–	–	–
λ *sidA*	30°C	+	+	+	+	+	–
(42°C)	37°C	+	+	+	+	+/–	–
	42°C	+	+	+/–	–	–	–

a. Temperature at which culture was maintained before experiment.

b. Temperature at which culture was streaked out on varying concentrations of ampicillin and a subjective estimate of viability made.

this hypothesis, since the temperature sensitive phenotype would allow selection for multiple lysogeny of λsidA. Lysogens of MM185 pBR325 (λ *sdaA*) were pre-grown either at 30°C or 42°C. Growth at the former temperature should select for single lysogens (since multiple lysogens in conjunction with the *dnaA46ts* allele would be cold-sensitive), while growth at 42°C should select for multiple lysogeny (i.e. suppression). These two types of lysogen, thus obtained, were streaked out on various concentrations of ampicillin plate, at both 30°C, 40°C and 42°C. A λ *NM616* MM185 pBR325 lysogen (obtained at 30°C) was streaked out as a control. The results of this experiment are shown in table 6.5. A clear correlation can be seen between the predicted amount of GroE (i.e. the growth temperature and the original lysogen type) and a decreasing ampicillin resistance. MM185 pBR325 λ *sdaA* lysogens obtained at 42°C exhibit a much reduced ampicillin resistance at all three growth temperatures, the effect being worse as the temperature is increased. Lysogens obtained at 30°C (and consequently not over-expressing *groE*) do not have a lowered ampicillin resistance at 30°C, although incapable of growth at the higher temperatures. The

MM185 pBR325 λ*NM616* control lysogens show this same effect; growth on high ampicillin at 30°C but no growth at the restrictive temperature.

However, this is not conclusive proof that the amount of GroE affects the level of ampicillin resistance, since an alternative hypothesis exists. The role of DnaA in the replication of pBR-type plasmids has not been fully resolved, with contradictory evidence concerning its involvement. (Data by Seufert and Messer 1987, Polaczek and Ciesla 1984, and Abe 1980 support a role for DnaA in replication of pBR322, while data by Frey *et al.* 1979, Hansen and Yarmolinsky 1986, and Minden and Marians 1985 has suggested that it may be unnecessary). Thus although DnaA does not appear to be essential for pBR-type plasmid replication, it seemed possible that in the *dnaAts* strain at 42°C, even though suppression by GroE was allowing chromosomal replication to take place, plasmid replication may be affected. Thus the lowered ampicillin resistance seen at this temperature could be due to a lower plasmid copy number rather than any specific effect of GroE. This seemed unlikely, but as a check a protein gel containing samples from MM185 pBR325 λ*sdaA* (30°C), MM185 pBR325 λ*sdaA* 42°C and MM185 pBR325 λ*NM616* (30°C) was run. This allowed a visual check on the levels of the chloramphenicol acetyl-transferase protein (CAT), which can be easily visualised as a strong band of about 26kd. Since this protein is encoded by the plasmid, any severe reduction in copy number should manifest itself as a reduction in the levels of this protein on an SDS-polyacrylamide gel. Ideally of course, the amount of β-lactamase proteins should be examined, although in contrast to the CAT protein these are not readily identifiable in a whole cell extract. The protein levels of these three strains are shown in figure 6.8, and it can be seen that there is no significant difference in the level of the CAT protein between strains, strongly indicative of an unchanged plasmid copy number. Additionally, the strong GroES and GroEL bands in the 42°C lysogen can be seen. It would thus seem likely that the presence of the 8.1kb *groE* DNA fragment in a high copy number, whether on a multiply-lysogenised phage or on a high copy plasmid, results in a lowering of the host cell resistance to ampicillin by a *trans*-acting effect. Furthermore, the fact that this reduction is temperature-dependent in nature would suggest it as being caused by the over-expression of one or more of the *groE* gene products.

FIGURE 6.8 Chloramphenicol Acetyl-Transferase levels in λ *sidA* lysogens

Position of Chloramphenicol Acetyl-Transferase protein marked with an arrowhead. 14-20% gradient gel.

Track 1: Molecular weight markers: Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsin Inhibitor, Lactalbumin

Track 2: MM185 dnaA46 pBR325 λ616 30°C lysogen

Track 3: MM185 dnaA46 pBR325 λsidA 30°C lysogen

Track 4: MM185 dnaA46 pBR325 λsidA 40°C lysogen

6.6.3 Discussion

What might be the molecular basis for this effect? While this work was in progress a paper by Kuriki (1987) demonstrated the involvement of the heat-shock response in determining the level of ampicillin resistance by post-translational control. He discovered that induction of the heat-shock response caused a reduction in the synthesis of β-lactamase, although since the amount of mRNA coding for this enzyme was not reduced this lead him to conclude that control at the level of translation was occurring. On the basis of the results obtained with GroE it seems plausible that the effect observed by Kuriki is due to induction of *groE* expression as part of the heat-shock response. Kuriki suggested that since β-lactamase is synthesised in an inactive form prior to cleavage and subsequent membrane translocation, possibly the rate of this processing is being affected. This would tie in with a role for GroE in which it might act to prevent the degradation of proteins at higher temperature; i.e. it affects protein processing. If this is the reason for the reduced ampicillin resistance in a GroE over-producing strain, then the levels of β-lactamase precursor forms should be higher compared to the active form. As a check on this, the protein gel shown in figure 3.4 was re-examined. This shows the plasmid-encoded proteins of pND5 and pBR325 in a minicell strain. However, both the total amounts and ratios of the 3 forms of the β-lactamase protein appear to be the same between the two plasmids, suggesting perhaps that a reduction in protein processing is not the cause of the reduced ampicillin resistance seen in a GroE over-producing strain. However, the situation in whole cells may be different; a more detailed study of processing of the β-lactamase precursors may lend an insight into this. Additionally, since the actual amounts of the β-lactamase proteins are the same in both pND5 and pBR325 the effect cannot be due to a reduced copy number of the former plasmid, and presumably is due to some specific interaction affecting β-lactamse function rather than its level.

6.7 Effect of pND5 on host-cell sensitivity to various membrane damaging agents

Since it was unclear how the above described effect is caused it was decided to investigate whether pND5-containing cultures exhibited generally reduced resistance to membrane damaging agents. If GroE somehow interacts in the outer membrane of *E.coli* in some undetermined manner, increased quantities may somehow result in the observed reduction in ampicillin resistance if some structurl balance is upset. This may be manifested by a generally increased susceptibilty to membrane damaging agents. To obtain a general indication of this, MM18 pND5 and MM18 pBR325 were spread onto L-agar plates in 3.5mls of top agar (0.1ml of stationary phase cells). 1.5cm diameter filter paper discs (Whatmann) which had been pre-soaked in a variety

of agents were then placed onto the upper surface of the top agar and the plates incubated overnight. The area of clearing (total and partial) around the disc was then measured the next day, in order to estimate any differences in susceptibility to agent under consideration. The membrane damaging agents under investigation included EDTA, SDS, Deoxycholate, NaOH and HCl. With the possible exception of EDTA, no apparent difference in sensitivity to any of these agents was noted. The MM18 pND5 culture did appear to be slightly more sensitive to EDTA, although it should be appreciated that such a test is crude and would not be expected to reveal subtle differences in sensitivity. Thus it would seem unlikely that over-production of the *groE* gene products has a general effect on cell membranes. Furthermore, since the level of β-lactamase protein appears to be the same in a GroE over-producing strain, this would perhaps suggest that the observed reduction in ampicillin resistance in such a strain is presumably due to some specific action of GroE affecting the functioning of the β-lactamase protein.

6.8 Effect of pND5 on host cell resistance to streptomycin

In view of the effect of pND5 on host cell resistance to the antibiotics ampicillin and nalidixic acid, it was decided to investigate whether the remaining antibiotic resistances of the host strain would be affected also. These were the streptomycin resistance due to the *rpsl* mutant allele in the MM-series of strains, together with the tetracycline resistance carried on pND5 itself. It has already been noted in chapter 4 that pND5 does not affect the host cell resistance to rifampicin.

MM18 pND5 and MM18 pBR325 were streaked onto L-agar plates containing various concentrations of one of the antibiotics, and incubated overnight at 30°C or 42°C. The viability and colony size were then compared. The results of this experiment are shown in table 6.6. No apparent effect on tetracycline resistance is seen. Somewhat suprising, in view of the fact that pND5 generally appears to have a deleterious effect on its host strain, it actually appears to cause an increase in host cell resistance to streptomycin, although pND5 when transformed into a non-mutant *rpsL* host cannot cause such a strain to become streptomycin resistant (data not shown). The action of the antibiotic streptomycin is to inhibit protein biosynthesis. Streptomycin has a target site within the 30S ribosomal subunit, and is inhibitory both in initiation and elongation, resulting in a mistranslation of the mRNA. Streptomycin resistant mutants are resistant by virtue of of the altered RpsL protein not binding the antibiotic. The fact that GroE over-producing strains exhibit an increased level of

TABLE 6.6 Effect of pND5 on the resistance of MM18 to tetracycline and streptomycin

PLASMID	GROWTH TEMP	ANTIBIOTIC CONCENTRATION (ug ml^{-1})				
Streptomycin						
		0	**200**	**400**	**800**	**1200**
pBR325	30°C	+++	+++	++	+	–
	42°C	+++	+++	++	+	–
pND5	30°C	+++	+++	+++	++	+
	42°C	+++	+++	+++	+++	+++
Tetracycline						
		0	**15**	**30**	**60**	**120**
pBR325	30°C	+++	+++	+++	++	+
	42°C	+++	+++	+++	++	+
pND5	30°C	+++	+++	+++	++	+
	42°C	+++	+++	+++	++	+

Strains were streaked to single colonies on L-agar plates (+ or – antibiotic) and incubated overnight (c.18hrs) before a subjective estimate of viability was made.

streptomycin resistance leads on to the intriguing possibility that it may be acting as an informational suppressor. This would conveniently explain how it is able to suppress *dnaAts* mutations. However, no direct evidence that it can act as a general informational suppressor has been obtained (see section 4.7). Since GroEL has been reported to be associated with ribosomes (Neidhardt *et al.* 1981), the extra GroEL protein may act to physically block streptomycin from interacting with the RpsL protein. Bearing in mind the role of GroE as a heat shock protein, an alternative explanation for the increased resistance to streptomycin in an over-producing strain may simply be that pND5-containing cells are better equipped to deal with the multitude of abberrant proteins likely to result from streptomycin induced mis-translation.

6.9 Summary

The results described in this chapter concern the phenotypic side-effects of maintaining the 8.1kb *groE* DNA fragment in a high copy number in the host strain. For the most part then it is not possible to say conclusively that such side-effects are due to over-expression of the *groE* genes, although the lack of other identified gene products on this fragment, together with the temperature related nature of most of these effects makes over-expression of the GroE proteins the most likely cause.

Plasmid pND5 appears to cause a general reduction in cellular growth rate. The degree of this effect is strain specific, and strains quickly seem to become 'adapted' with an essentially normal growth rate. The most likely cause of this effect is probably the metabolic strain placed on the host cell due to over-production of a single protein species.

Plasmid pND5 also appears to cause an adverse effect upon host-cell resistance to the antibiotic nalidixic acid, again in a temperature dependent way. This may reflect an interaction between GroE, gyrase and nalidixic acid during DNA replication, and may be relevant to the process by which GroE is able to suppress *dnaAts* mutations. The effect of pND5 upon growth in media containing nalidixic acid is to cause a long lag before growth resumes; if the strain is pre-grown in nalidixic acid-containing media this lag is not seen.

An alternative hypothesis for this effect is that nalidixic acid treatment results in over-expression of the *groE* genes from the high copy plasmid and hence causes 'GroE poisoning'. This seems unlikely, since pND5-containing strains do not appear to be significantly affected by either ethanol shock or UV irradiation, the former of which at least is more efficient at inducing *groE* expression than nalidixic acid.

Both plasmid pND5 and λsidA result in a lowered host-cell resistance to the antibiotic ampicillin. This is in agreement with results obtained by Kuriki (1987) in which he showed that the amount of β-lactamase activity is decreased following heat-shock, although he did not identify the *groE* gene products as being responsible. This may reflect an involvement in post-translational control by GroE. An alternative hypothesis -that increased amounts of the GroE proteins results in a generally increased sensitivity to membrane damaging agents- appears to be unfounded.

The streptomycin resistance of an already resistant strain appears to be increased in a strain carrying pND5, possibly suggesting an involvement in informational suppression or the cellular response to the multitude of abberrant

proteins likely to be produced under such conditions. On the basis of previous data it was concluded that informational suppression was unlikely to be taking place. However, the results presented in this chapter, although possibly unrelated to the direct mechanism by which over-expression of *groE* is able to suppress *dnaAts* mutations, have produced data in agreement with two of the most likely possibilities; that of a direct protein-protein interaction during the initiation events, and that of an effect upon processing of the DnaAts protein following temperature shift and thermal denaturation.

CHAPTER 7

The isolation and characterisation of an *E.coli* chromosomal mutation which reduces plasmid copy number

7.1 Introduction

The experiments detailed in this chapter describe the isolation and characterisation of a chromosomal mutation which affects the copy number of certain plasmid replicons. As a result of this phenotype the mutation is referred to as *pcn* (for reduction in plasmid copy number). The mutation was originally isolated and characterised as causing a reduction in the copy number of the pMB1-derived cloning vehicle pBR325 (Bolivar 1978); pMB1 itself is structurally and functionally very closely related to the well characterised plasmid ColE1 (see Lacatena and Cesareni 1983).

Replication of ColE1-type plasmids is entirely dependent upon *E.coli* encoded proteins, no plasmid specified proteins being required either *in vivo* or *in vitro* (Tomizawa *et al.* 1974). The range of host proteins normally required includes DNA polymerase I (the product of the *polA* gene), RNA polymerase, RNAase H, DNA polymerase III, and the products of the *dnaB, dnaC, dnaG* and *dnaZ* genes (Kingsbury and Helinski 1970, 1973b, Cozzarelli *et al.* 1968, Staudenbauer 1976, Itoh and Tomizawa, 1978, 1980). This list should not be considered exhaustive. The role of the DnaA protein in ColE1 DNA replication is unclear; it appears to participate under certain conditions, although at the same time it is not essential (see Seufert and Messer 1987). One of the features of the *pcn* mutation is the lack of a phenotypic effect on its host strain; most of the above gene products (with the possible exception of RNAase H and DNA polymerase I) are normally required for *E.coli* DNA replication, and mutants which affect plasmid replication would also have an adverse effect on host cell growth. The absence of deleterious effects gave grounds to believe that a new class of mutant had been isolated, worthy of further study.

During the course of this work, a similar low copy mutation called *pcnB* was isolated by Lopilato *et al.* (1986). A strain carrying this mutation was obtained and it was shown to be allelic to that described in this work. For that reason, the copy number gene described hereafter is referred to as *pcnB* or simply *pcn*.

Since *pcn* was originally thought only to effect the replication of ColE1-type plasmids, it was intended to present a detailed description of the replication and control mechanisms of ColE1. These could then have been compared to the corresponding mechanisms for a variety of other replicons to see which mechanisms were unique to ColE1. It was hoped this might provide a clue to the possible function of the *pcn* gene product. However, the more recent finding that the *pcn* gene product is required by a number of different plasmids suggests that its role in the cell might be of a more general nature than was previously supposed. For this reason I

shall describe the identification and characterisation of the *pcn* gene product, and then go on to discuss the replication and control mechanisms of both Pcn-requiring and Pcn-independent replicons. This may allow identification of features restricted to affected plasmids and hence provide an indicator towards the role of this protein in the control of plasmid copy number.

7.2 Identification of *pcn* as a copy number mutant

7.2.1 Isolation of *pcn*

The cold sensitivity caused by over-expression of the *groE* genes in combination with certain *dnaAts* alleles led to the isolation of the *pcn* mutant described in this study (see also section 4.4.2). A culture of MM185 (*dnaA46ts*) pND5, which had been grown at 30°C for several hours was streaked onto an L-agar plate and incubated overnight at 30°C. Tetracycline at 5μg ml^{-1} was included in the media throughout to maintain selection for the plasmid. The original purpose of doing this was to verify the phenotype of the cultures which had been used to measure the growth rates of MM185 pBR325 and MM185 pND5. To do this, an aliquot from each growth temperature was streaked out at the end of the day to check for antibiotic resistances, temperature sensitivity and cold sensitivity. On the MM185 pND5 plate incubated at 30°C it was noticed that several large colonies had appeared against the general background of poorly growing colonies resulting from the cold sensitivity conferred by pND5. These large colonies were restreaked at 30°C, 40°C and 42°C together with 'normal sized' (cold sensitive) colonies and MM185 pBR325 as a control. The colony sizes at each of the temperatures are shown in table 7.1. It is apparent that the large colonies picked from the 30°C plate are now less able to grow at the higher temperature compared to the cold sensitive parent. The most likely explanation for this was thought to be that a spontaneous mutation had occurred which reduced the ability of pND5 to suppress *dnaAts* while concomitantly resulting in a loss of cold sensitivity. The mutant was considered deserving of further study, since it was hoped that it might shed some light on the cold sensitive phenotype of *groE* over-expression (can cold sensitivity and suppression of *dnaAts* be separated for example), and secondly, mutations which affected the ability of *groE* to suppress might give some insights into the mechanisms involved. Since all the large (non-cold sensitive) variants of MM185 pND5 had been isolated from the same culture, and since each behaved identically, it was considered likely that they were all derived from the same original mutant. Therefore only one was saved; it was called JM21 pND5.

TABLE 7.1 Cold sensitivity of *pcn* strain compared to MM185 pND5

STRAIN	30°C	40°C	42°C
COLONY DIAMETER (mm)			
MM185 pND5 *pcn*	1.0	1.0	0.1
MM185 pND5	0.3	1.5	1.2

Cultures streaked to single colonies and incubated overnight (c. 18hrs) at the appropriate temperature whereupon colony diameter was measured.

7.2.2 The mutation in JM21 is chromosomal in origin

The mutation in JM21 could be either plasmid or chromosomal in origin. Initially, it was thought possible that a deletion derivative of pND5 might have taken over the population, since growth at 30°C was obviously selecting against *groE* over-expression due to the resulting cold sensitivity. Such deletion derivatives had previously been seen in populations of MM18 pND5 when selection for over-expression of *groE* was relaxed. As an initial check of this, JM21 pND5 was streaked on both ampicillin and tetracycline selective plates in order to see if any antibiotic resistances had been lost. Growth was observed on both types of selective medium, indicating that a deletion, if present, had not markedly affected the vector fragment of the plasmid. The plasmid contained in JM21 was then extracted and analysed alongside pND5 by agarose gel electrophoresis. Both plasmids appeared identical in size (data not shown) which suggested that if a deletion had occurred then it must be very small. However, it seemed possible that a point mutation affecting either the function or expression of the plasmid copy of the *groE* genes might have occured. Accordingly, the 'pND5' isolated from JM21 was used to back-transform MM185, and the transformation mix plated at 37°C. Colonies obtained were streaked at 30°C, 40°C and 42°C, together with MM185 pND5 and JM21 pND5 as controls. The results are shown in table 7.2. The MM185 colonies transformed with the 'pND5' isolated from JM21 behave exactly as did the original MM185 pND5, suggesting that the phenotype seen with JM21 pND5 is due to a chromosomal change, and not due to any change in the plasmid.

TABLE 7.2 Transformation of pND5 plasmid isolated from JM21

STRAIN	30°C	40°C	42°C
COLONY DIAMETER (mm)			
JM21 pND5	1.0	1.0	0.1
MM185 pND5[a]	0.3	1.5	1.2
MM185 pND5	0.3	1.5	1.2

a. MM185 transformed with the pND5 isolated from JM21 pND5.

7.2.3 Curing JM21 of pND5

As a check to ensure that the phenotype exhibited by JM21 was exclusively due to a chromosomal mutation, and not due to a simultaneous change to the plasmid also, it was decided to cure JM21 of pND5, re-transform with a 'known' pND5 and check to see if the same phenotype had returned. Since pND5 was known to reduce nalidixic acid resistance (see chapter 6), it was decided to grow JM21 pND5 in the presence of L-broth containing nalidixic acid at 50µg ml^{-1} in an attempt to increase curing of the plasmid. A single colony was innoculated into 5ml of L-broth and grown at 30°C to stationary phase. An identical culture without nalidixic acid was included also. A 10^{-6} dilution was made into bacterial buffer, and 0.2ml of this was plated onto L-agar plates and incubated at 30°C to give single colonies. These were then patched onto L-agar and L-agar plus tetracycline to look for plasmid loss. The results are shown in table 7.3. Somewhat suprisingly, both cultures exhibited an equally high rate of plasmid loss. While growth in nalidixic acid containing medium was expected to select against pND5, in its absence there was expected to be no selection against the plasmid, since pND5 does not appear to reduce the growth rate in JM21 at 30°C. This seemed to suggest that the mutation in JM21 might be affecting plasmid copy number.

JM21 was then transformed with a 'known' pND5 plasmid, as well as pBR325 as a control. This retransformed JM21 pND5 behaved as had the original isolate (i.e. unable to grow at 42°C and not cold sensitive at 30°C), indicating that the phenotype seen in JM21 is purely chromosomal in origin.

TABLE 7.3 Stability of pND5 in JM21 and MM185

STRAIN	LA	LAtet
GROWTH OF PATCHES		
JM21 pND5 30°C	100	73
JM21 pND5 30°C nal[a]	100	72

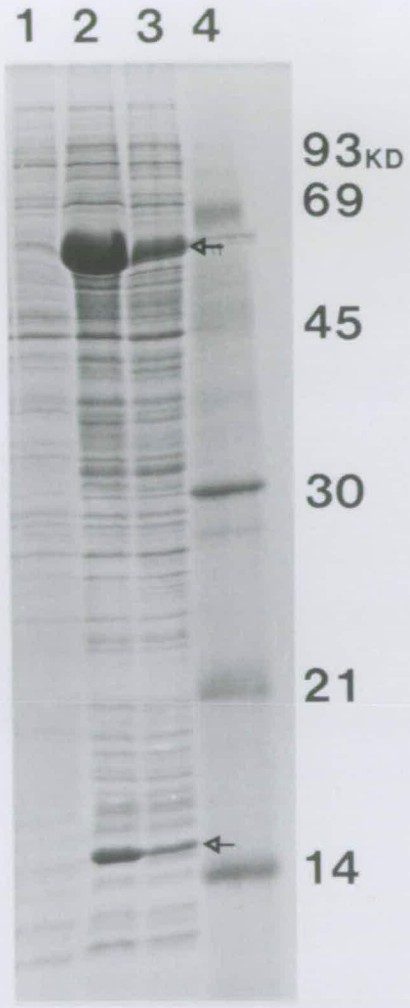
a. Growth in medium containing nalidixic acid prior to assay for plasmid loss.

7.2.4 Examination of GroE protein levels in JM21 pND5

The fact that cold-sensitivity appeared to be correlated to some degree with the extent of GroE over-production (section 4.4.3) suggested that the concommitant loss of cold sensitivity and the ability to suppress up to 42°C might be due to a lower level of GroE in JM21 pND5 as compared to MM185 pND5. To examine this, whole cell proteins in MM185 pND5 and JM21 pND5 were separated by SDS-polyacrylamide gel electrophoresis and the GroE levels compared. Both cultures were grown at 37°C prior to sampling, and the amount loaded on the gel was the same in each case. This is shown in figure 7.1. The GroE protein levels are clearly much reduced in JM21 pND5 (although still higher than in a strain lacking pND5), demonstrating that the observed phenotype is most probably due to a reduction in GroE levels in the mutant.

Two obvious explanations presented themselves as to why GroE protein levels are reduced in JM21. Firstly, a mutation which affects control or expression of the *groE* genes could be responsible (this would have to be a *trans* acting mutation since it has already been demonstrated that the mutation is chromosomally located). Secondly, the plasmid copy number could be reduced in this strain, thereby reducing the *groE* gene dosage. To test the former hypothesis a lawn of JM21 was plated at 42°C and λ *groE* (λsidA; section 3.2) was spotted on to test for suppression. Colonies appeared in the area of the phage spot, indicating that the mutation in JM21 does not affect the ability of *groE* to suppress *dnaAts per se*. Instead, it appeared likely to be the result of a gene dosage effect caused by a lower plasmid copy number in JM21 rather than any effect upon *groE* expression which was the cause of the observed phenotype.

FIGURE 7.1 GroE protein levels in JM21 pND5 and MM185 pND5



Position of GroES and GroEL proteins marked by an arrowhead. Cultures grown up at 40°C prior to loading on gel. 14-20% gradient gel.

Track 1: MM18 dnaA$^+$

Track 2: MM185 dnaA46 pND5

Track 3: JM21 dnaA46 pND5

Track 4: Molecular weight markers: Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsin Inhibitor, Lactalbumin

7.2.5 Measurement of plasmid copy number in JM21

The high rate of pND5 curing together with the lowered *groE* protein levels seen in JM21 pND5 suggested that the observed phenotype was the result of a lowered plasmid copy number in this strain. Several experiments were conducted to test this. Firstly, the ampicillin resistance of JM21 pBR325 was compared to that of the parental, MM185 pBR325. A stationary phase culture was diluted down to single colonies and plated on various concentrations of ampicillin at 30°C, and the number of colonies at each level compared. Table 7.4a shows that JM21 pBR325 is only resistant to 800µg ml^{-1} ampicillin compared to a resistance level of 2400µg ml^{-1} for MM185 pBR325. Assuming a linear response of ampicillin resistance to gene dosage (Neil Willets, pers. comm. to Sullivan 1983), this suggests a reduction in copy number of about two thirds in JM21.

TABLE 7.4 Ampicillin resistance of plasmids pND5, pHR3 and pBR325 in JM21 and MM185

STRAIN	PLASMID	AMPICILLIN CONCENTRATION ug ml^{-1}						
		0	400	800	1600	2000	2400	2800
NUMBER OF COLONIES								
(A)								
JM21	pBR325	52	48	48	-	-	-	-
MM185	pBR325	40	35	38	37	34	37	-
(B)								
JM21	pND5	69	67	-	-	-	-	-
MM185	pND5	31	32	-	-	-	-	-
JM21	pHR3	50	57	48	51	46	49	-
MM185	pHR3	74	70	71	68	65	60	-

However, it should be remembered that a reduction in ampicillin resistance need not necessarily be due to a reduction in plasmid copy number. A chromosomal mutation which affects expression of the ampicillin resistance gene or affects its mode of action in some way could give similar results. An example of this is seen when examining the ampicillin resistance conferred by pND5 or pHR3 in JM21. Plasmid pHR3

contains the *rpoBC* genes cloned into pBR322 (Newman and Hayward 1980). It was transformed into JM21 so that complementation of *pcn* by λ*polA* could be tested (described in section 7.3.2). As this phage carries the tetracycline resistance gene, a tetracycline sensitive plasmid (pHR3) was desired to enable an easy selection for lysogens. (The ampicillin resistance levels conferred by a plasmid in a λ*polA* background could be compared to those in a non-lysogen to see if complementation was occurring. Plasmid pBR325 contains the tetracycline resistance gene; it would be more difficult to obtain a λ*polA* lysogen because tetracycline resistance could not be used to select for lysogens. For this reason JM21 pHR3 was used; it is ampicillin resistant but tetracycline sensitive, so a λ*polA* lysogen could be easily selected). However, it appeared that pHR3 has just as high a copy number in JM21 as in MM185 (ampicillin resistance up to 2400μg ml^{-1}; see table 7.4b). Initially it was thought that pHR3 might be complementing the low copy mutation of JM21. Since RNA polymerase is involved both in the initiation of DNA replication at *oriC* (Zyskind *et al.* 1977) and at the origin of ColE1 (Sakakibara and Tomizawa 1974), it seemed possible that a mutation in one or other of the *rpoB* genes may conceivably effect plasmid copy number. As an initial test of whether pHR3 did have a reduced copy number in JM21, overnight cultures of JM21 pHR3 and MM185 pHR3 were diluted 1:1000, grown to stationary phase, diluted to single colonies and patched to test for plasmid loss as previously described. JM21 pBR325 and MM185 pBR325 were included as controls. Examining table 7.5 it appears that pHR3, in common with pBR325 does indeed cure at a faster rate in JM21, although the plasmid is not totally stable in the MM185

TABLE 7.5 Stability of pHR3 and pBR325 in JM21 and MM185

STRAIN	LA	LAamp
GROWTH OF PATCHES		
MM185 pHR3	100	98
JM21 pHR3	100	86
MM185 pBR325	100	100
JM21 pBR325	100	84

TABLE 7.6 Plasmid copy number in JM21 measured by quantitative hybridization

STRAIN	PLASMID	RELATIVE LEVEL NORMALISED TO MM185 pBR325 = 1.0	LEVEL IN JM21 AS A PERCENTAGE OF LEVEL IN MM185
MM185[a]	pND5	0.55	65%
JM21[a]		0.36	
MM185[b]	pBR325	1.00	24%
JM21[b]		0.24	
MM185[b]	pHR3	0.81	30%
JM21[b]		0.24	

a. Cultures grown at 37°C.
b. Cultures grown at 30°C.

background either. This suggested that the copy number of pHR3 is indeed reduced in JM21, despite the high level of penicillin resistance seen. The opposite situation is seen with plasmid pND5, where low ampicillin resistance is seen in both JM21 and MM185, suggesting (on the basis of ampicillin resistance alone) a low copy number in both backgrounds. To obtain a more accurate determination of the respective copy numbers it was decided to probe directly to the plasmids using quantitative hybridisation (described in chapter 8). The results of these hybridisations, with the data normalised to to a copy number of pBR325 in MM185 = 1.0 is shown in table 7.6. The copy number of all three plasmids does appear to be reduced in JM21; that of pHR3 and pBR325 being reduced to about one third of the figure seen in MM185. This is in agreement with the ampicillin resistance figures noted when comparing MM185 pBR325 and JM21 pBR325. However, although the copy number of pHR3 is reduced in JM21, the level of ampicillin resistance conferred is as high as that of pBR325 in MM185. This may be due to the presence of a promoter in the insert fragment reading into the ampicillin resistance gene and thus increasing its expression, such that the copy number appears artificially high. However, the question of why the ampicillin resistance is not even higher in the *pcn*$^+$ background requires consideration. It may be that the maximum level of ampicillin resistance possible is in the region of 2400μg ml^{-1}; any further production of β-lactamse may not be reflected by an

increased resistance to the antibiotic. The converse situation is seen with pND5, where a reduction in copy number is not accompanied by a further reduction in the already low level of ampicillin resistance. In fact, pND5 appears to confer a level of ampicillin resistance in both JM21 and MM185 incommensurate with its copy number. This appears to be due to over-expression of *groE* reducing ampicillin resistance without actually effecting copy number. Since this effect on ampicillin resistance is also seen in λ*sidA* lysogens containing pBR325 (see section 6.6 for more details), this is obviously a *trans* acting effect, and not due to transcriptional effects such as probably occur in pHR3. Thus while in some instances the level of ampicillin resistance conferred by a plasmid can be taken as being representative of its copy number, the limitations of such an approach should be appreciated if aberrant and misleading interpretations are not to result.

7.3 The mutation in JM21 is not in the *polA* gene

7.3.1 Measurement of the UV sensitivity of JM21

The above data demonstrates that JM21 has a chromosomal mutation which reduces the copy number of pBR-type plasmids and their derivatives. The most obvious question at this stage was to identify which gene is responsible for this phenotype. Since JM21 grows as well as the MM185 parental strain (table 7.1), the mutation at this stage could only be classifed as effecting plasmid copy number without any noticable affects on the host strain. It was considered most likely that this low copy phenotype was due to a mutation in *polA*, which codes for DNA polymerase I, since such mutants exhibit a similar phenotype to that of JM21; namely a reduction in plasmid copy number without any overt effect on host cell physiology. A common phenotype of *polA* strains is their increased sensitivity to UV light, so it was decided to compare the UV resistances of JM21, MM20 (an otherwise isogenic *polA* strain), and MM185 as a positive control. Cross-streaks and exposure to different intensities of UV light were performed, and the viability of each strain estimated at each intensity. These results are shown in table 7.7, and reveal no difference in UV sensitivity between JM21 and the parent MM185; that of the *polA* strain (MM20) is markedly increased in comparison.

TABLE 7.7 UV sensitivity of JM21

STRAIN	EXPOSURE TO UV IRRADIATION (Seconds) 0	10	30	60
VIABILITY				
MM185	+++	+++	+++	+++
JM21 *pcn*	+++	+++	+++	+++
MM20 *polA*	+++	++	+	–

a. Subjective estimate of viability, compared to MM185 at 0s exposure = +++.

7.3.2 Attempted complementation of *pcn* using λ*polA*

However, in spite of the above result it still appeared possible that JM21 was a leaky *polA* mutant. The mutation in JM21 only results in a lowering of plasmid copy number; that in MM20 prevents the maintenance of pBR-type plasmids at all (Jenkins *et al.* 1986). Therefore it appears reasonable to expect that if JM21 is a leaky *polA* mutant then it should be less sensitive to UV than MM20 is.

As a more definitive test of whether JM21 is a *polA* mutant, complementation using λ*polA* (λ851) was attempted. This phage carries tetracycline resistance; therefore a tetracycline sensitive pBR-type plasmid was desired to enable easy selection for lysogeny. JM21 was originally transformed with pHR3 for this purpose, but as described earlier, the level of ampicillin resistance conferred by this plasmid does not appear to be proportional to the copy number. Instead, λ*polA* was spotted onto a growing lawn of JM21 pBR325, and cells streaked out the following day from the area of the turbid plaque. Individual colonies were then tested for lysogeny by streaking across an L-agar plate and spotting λ*vir* and λ46 (λclear) onto the streaks. Lysogens were lysed by λ*vir* but not λ46, while non-lysogens were lysed by both phage. These lysogens, together with JM21 pBR325 and MM185 pBR325 as controls, were then streaked on high ampicillin (2400μg ml^{-1}) and low ampicillin (50μg ml^{-1}) plates. In contrast to MM185 pBR325, the λ*polA* lysogens were unable to grow on high ampicillin plates, indicating either that *pcn* is not an allele of *polA*, or alternatively, that it is a dominant *polA* mutation.

7.4 Transducing JM21 to *dnaA*$^+$: construction of JM18

To increase the ease of working with the low copy strain, and in order to see if the low copy phenotype was in any way due to the *dnaAts* genotype of JM21, it was decided to transduce the strain to *dnaA*$^+$ using a lysate made on MM18. MM18 is the parental strain of MM185 (MM18 *asn*$^+$ *dnaA46ts*). JM21 pBR325 was therefore transduced to temperature resistance, and the colonies screened for the *asn*$^-$ phenotype of MM18 in order to isolate true *dnaA*$^+$ transductants and not revertants or *dnaA* suppressors. About 25% of temperature resistant transductants simultaneously became *asn*$^-$. Several of these were tested for the low copy phenotype by streaking out onto high and low ampicillin plates, with MM18 pBR325 as a control. In contrast to MM18 pBR325, none of the transductants tested was able to grow on high ampicillin, indicating that the *pcn* phenotype of JM21 is unconnected with its *dnaAts* genotype. This *dnaA*$^+$ *asn*$^-$ version of JM21 pBR325 was called JM18 pBR325, since it is isogenic with MM18 except for the *pcn* mutation. JM18 pBR325 was cured of its plasmid by diluting 1:100 into L-broth and growing to stationary phase. Single colonies were obtained and tested for plasmid loss as described earlier, with the result that 3 out of 100 colonies tested had lost the plasmid. This cured version was simply called JM18.

7.5 Determination of the best selection system for the *pcn* marker

Since a direct measurement of plasmid copy number by quantitative hybridisation is a fairly involved process, it was considered impractical as a means of screening the large number of exconjugants and transductants likely to be produced during the mapping of a chromosomal locus. Therefore in order to map *pcn* a suitable selection system was required. Two possibilities presented themselves. The first was to use the non-cold sensitive/reduced viability at high temperature phenotype of JM21. The lack of cold sensitivity exhibited by JM21 pND5 could be detected as larger colonies when steaked at 30°C. However, this only allowed for a screening of the marker rather than direct selection; additionally, it was felt that comparing colony sizes might be too open to interpretative errors. Direct selection for growth at 42°C proved to be unsatisfactory also. Although JM21 pND5 grew less well at this temperature than MM185 pND5, again the differences were not as great as might be hoped for. Additionally, fluctuations in incubation temperature would probably lead to misleading results.

The second possibility was to make use of the different levels of antibiotic resistance conferred by pBR325 in the mutant and non-mutant backgrounds. This

appeared to be a more useful selective system, since the presence or absence of growth rather than differing degrees of growth are being compared. Plasmid pBR325 carries resistance genes to ampicillin, tetracycline and chloramphenicol. Therefore it was decided to compare the resistance levels of JM18 pBR325 and MM18 pBR325 to these antibiotics in order to determine which selection procedure showed the biggest difference between the two strains. Overnight cultures of both strains were diluted down to give single colonies and plated out on different concentrations of chloramphenicol, ampicillin, or tetracycline plates. The number of colonies obtained at the different antibiotic concentrations are shown graphically in figure 7.2. It is clear that ampicillin appears to be the best selective system, the resistance figures being proportional to the plasmid copy number as determined by quantitative hybridisation. Tetracycline is next best, although the difference between the strains is much less pronounced. No detectable difference in chloramphenicol resistance between JM18 pBR325 and MM18 pBR325 is seen, indicating that resistance to this drug is fairly non-linear with respect to gene dosage. These results suggested the measurement of ampicillin resistance as the best technique for detecting the presence/absence of the low copy mutation, and this was the method used throughout the mapping of this marker.

However, despite the large difference in ampicillin resistance between MM18 pBR325 and JM18 pBR325 it was found impossible to select directly for high copy recombinants amongst a population of low copy cells (such as would occur in a transduction for example). Since these low copy mutants exhibited some ampicillin resistance, only when low numbers of cells were plated (e.g. 10^3 - 10^4 cells per plate) could the two colony types be reliably distinguished. Instead, exconjugants or transductants selected for a possible linked marker were isolated; these were then screened for the presence of the *pcn* marker. The best way to do this was found to be purification of a clone followed by growth to stationary phase in liquid media. A 10^{-6} dilution of this culture was then made and 50µl plated onto a quarter of an L-agar plate containing ampicillin at either 50µg ml^{-1} or 1200µg ml^{-1}. This allowed the screening of up to four clones per plate, with low copy mutants being unable to grow on the high ampicillin plate whilst growing on the low ampicillin plate.

FIGURE 7.2 Ampicillin, Chloramphenicol, and Tetracycline resistance curves of JM18 pBR325 and MM18 pBR325

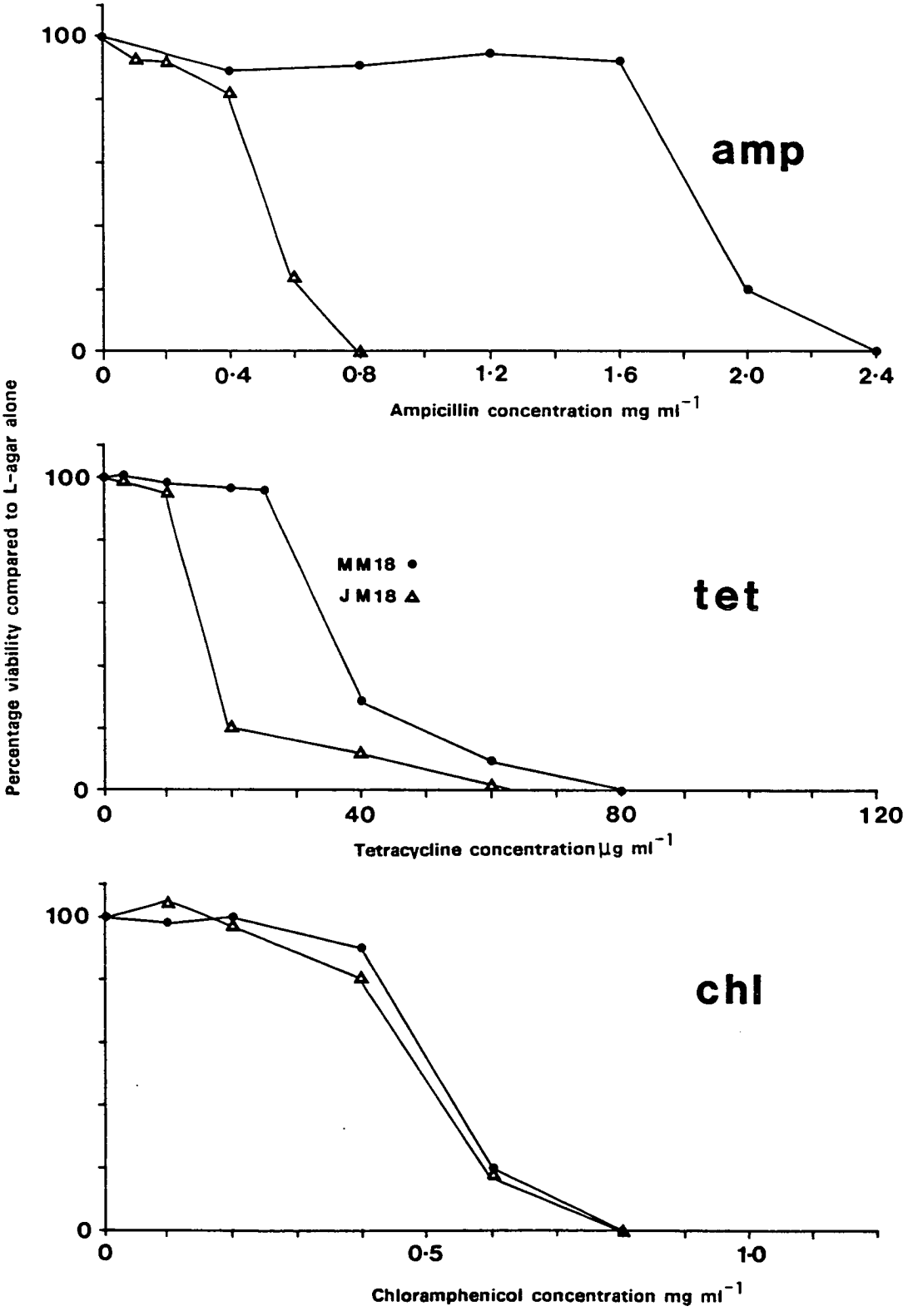
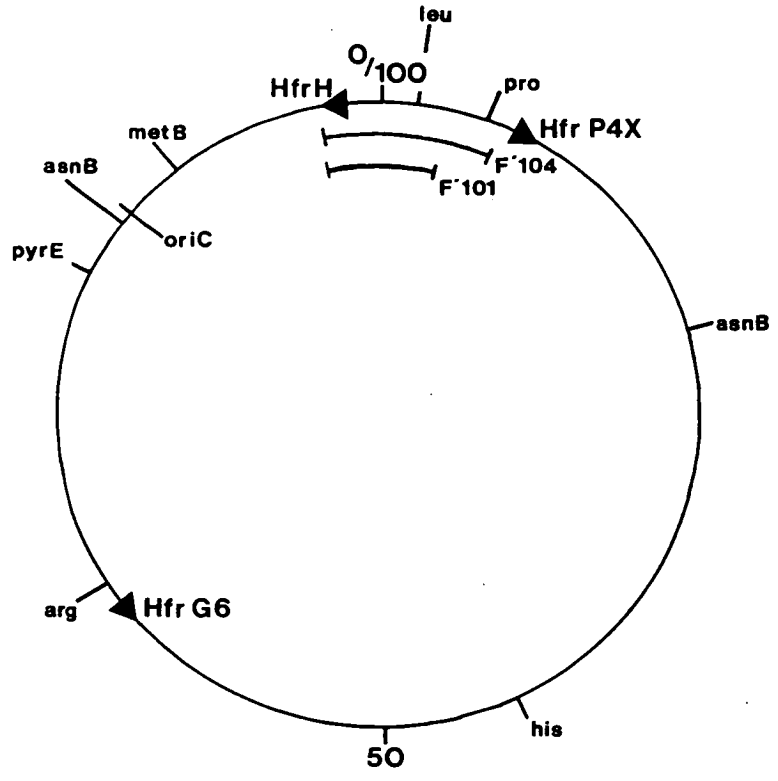


FIGURE 7.3 Hfrs and F-Primes used in mapping the *pcn* locus



Data taken from Bachmann (1983) and Miller (1972).

7.6 Mapping of the *pcn* locus

The Hfr strains and F-primes used in the mapping of *pcn* are shown in figure 7.3. Initially HfrG6 was mated with JM18 pBR325 and various nutritional markers were selected and exconjugants (16 of each) were tested for co-inheritance of *pcn* (resistance to high ampicillin). Results are shown in table 7.8a. Approximately 70% coinheritance with *leu* is seen; no linkage was found with the other markers tested. When *leu*$^+$ exconjugants of HfrH were tested, 15 out of 24 (62.5%) coinheritance with *pcn* was observed (table 7.8a). This suggested that *pcn* could be located between the origin of transfer of HfrH (98.5 minutes) and at a point near *leu* on the genetic map (2 minutes). Since *polB* (DNA polymerase II) maps at 2 minutes on the genetic map (Bachmann 1983), this appeared to be a possible candidate for *pcn*. Accordingly, JM18 pBR325 was transduced to *leu*$^+$ using a P1Kc lysate on W3110, and 100 transductants

TABLE 7.8 Mapping of the *pcn* mutation

Hfr or F-Prime	Marker	Linkage
(A) Hfr and F-Prime linkage with *pcn*		
Hfr G6	*leu*	70%
Hfr H	*leu*	62%
Hfr P4X	*leu*	92%
F'104	(*thr-pro*)[a]	100%
F'101	(*thr-leu*)[a]	0%
	Marker	**Cotransduction**
(B) Cotransduction with *pcn*		
	leu	0%
	folA	0%
	thr	0%
	(*proA-dnaE*)[a]	0%
	panB	72%
	dapD	37%

a. Signifies that region between these markers was tested to see if pcn was contained within.

were screened for cotransduction of *pcn*$^+$. None of the *leu*$^+$ transductants had simultaneously become *pcn*$^+$ (table 7.8b). Thus *pcn* could either lie between 98.5–0.5 minutes, or from 3 minutes onwards, assuming that P1 packages about 2 minutes of the *E.coli* chromosome (Masters 1985). This clearly indicated that *pcn* could not be *polB*, although other markers concerned with DNA replication which could be *pcn* included *dnaC, dnaT* (99 minutes), *dnaE* (4.5 minutes) and *rnh* (5.5 minutes) (Bachmann 1983). The positions of these and all other markers used in the mapping of *pcn* are shown in figure 7.4. JM18 was then transduced to Trimethoprim resistance (*folA*) using a P1Kc lysate made on N1270, which is also *thr*$^-$. Of the 46 *folA* transductants screened, 18 became *thr*$^-$, while 28 remained *thr*$^+$. All transductants remained *pcn*$^-$ (table 7.8b), indicating that *pcn* did not lie between *thr* and *folA* (0–1 minute). This *thr*$^-$ *folA* version of JM18 pBR325 was called JM19 pBR325. JM19 pBR325 was

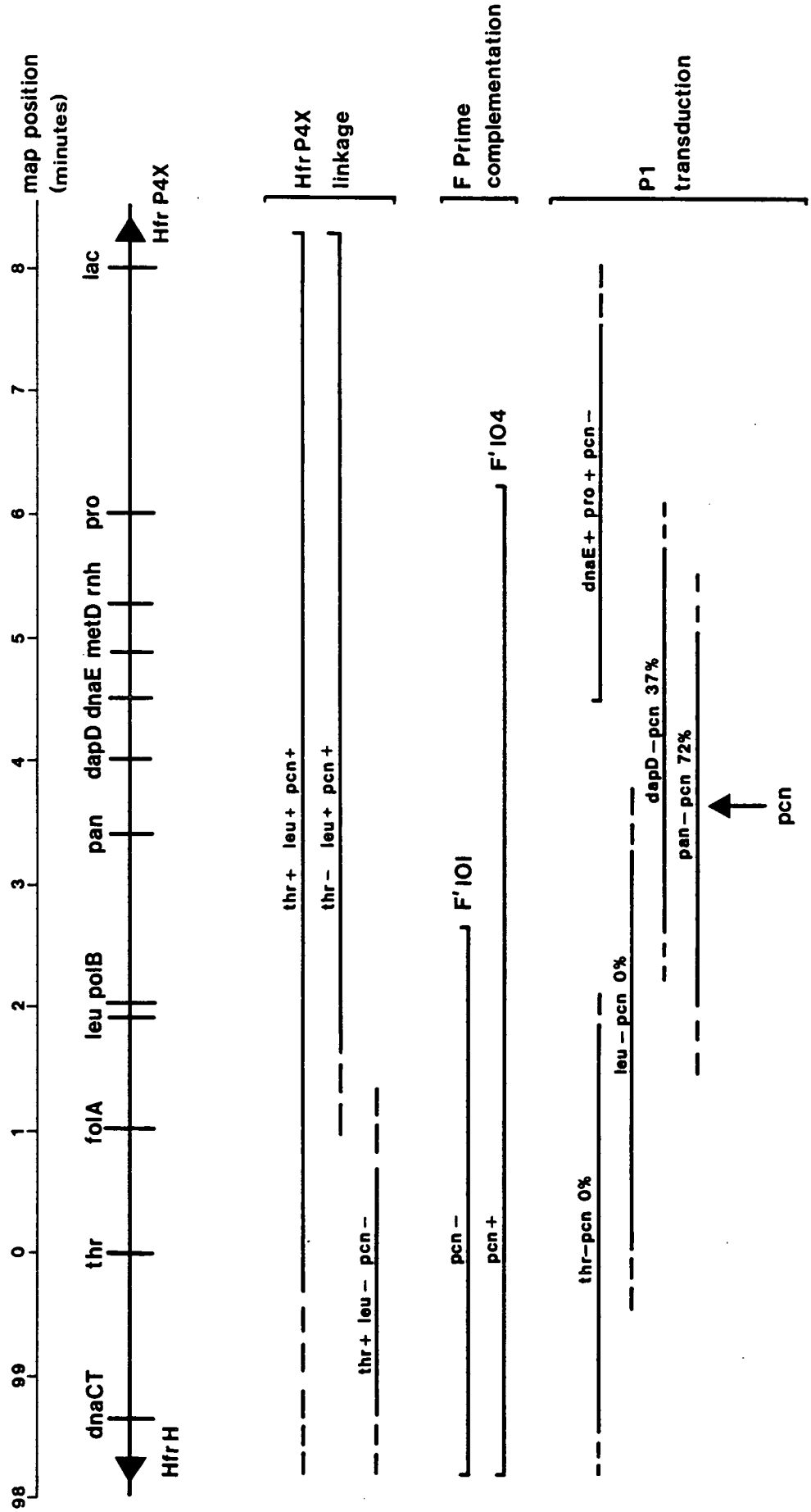
transduced to *thr*$^+$, maintaining selection for trimethoprim resistance in order to select for transductants which had inherited donor DNA to the left of *thr* (towards *dnaCT*). Of the 100 *thr*$^+$ transductants tested, none was able to grow on high ampicillin, suggesting that *pcn* was not *dnaC* or *dnaT* (table 7.8b).

Thus at this stage it appeared likely that *pcn* mapped to the distal side of *leu*. Although the high frequency of coinheritance with *leu* suggested that *pcn* was probably located fairly close by, it could only be said with certainty that *pcn* was located distal to 3 minutes on the genetic map. In order to give a more delimited location for *pcn*, HfrP4X was mated with JM19 pBR325. HfrP4X has an origin of transfer between *pro* and *lac* (6-8 minutes; Miller 1972). Both *leu*$^+$ and *thr*$^+$ exconjugants were selected individually, and then tested for coinheritance with *pcn* and the unselected nutritional marker. Some exconjugants which remained *thr*$^-$ simultaneously became *pcn*$^+$, confirming that *pcn* did not lie between 98.5-0.5 minutes. Furthermore, since HfrP4X could transfer *pcn*, this proved that its location must be between 3-8 minutes (see also figure 7.4). Again, a high frequency of coinheritance with *leu* was seen (92%; table 7.8a).

In order to further delimit the map position of *pcn*, while investigating whether it could be complemented *in trans* (i.e. *pcn* is not a dominant mutation), the F primes F'101 and F'104 were mated into JM19 pBR325, selecting for *thr*$^+$ and *leu*$^+$ by plate matings. Several exconjugants of each type were tested; all F'104 exconjugants had become *pcn*$^+$, while all F'101 exconjugants remained *pcn*$^-$. Since F'104 carries *proA* but not *proB* (Miller 1972), this further delimited *pcn* to between 3-6 minutes (the location of *proAB* on the genetic map; Bachmann 1983). Additionally, this result demonstated that *pcn*$^-$/*pcn*$^+$ merodiploids are phenotypically *pcn*$^+$.

The region between *dnaE* (4.5 minutes) and *pro* (6 minutes) was examined next, using strain KH1331 pBR325, which is *dnaEts, pro*$^-$. This strain proved relatively difficult to work with, since it reverted to temperature resistance at a high frequency. Accordingly, rather than transduce directly to *dnaE*$^+$,it was decided to transduce KH1331 pBR325 to *pro*$^+$ using a P1Kc lysate made on JM18 and screen for temperature resistant colonies. This should result in transductants with the DNA between *pro* and *dnaE* replaced, which could then be screened for the presence of the *pcn* marker. Because of this, it was important to know what the ampicillin resistance conferred by pBR325 in this background normally is. Since KH1331 is temperature sensitive, the ampicillin resistance of KH1331 pBR325 was measured at 30°C, with JM18 pBR325 as a

FIGURE 7.4 Location of *pcn* on the *E.coli* genetic map



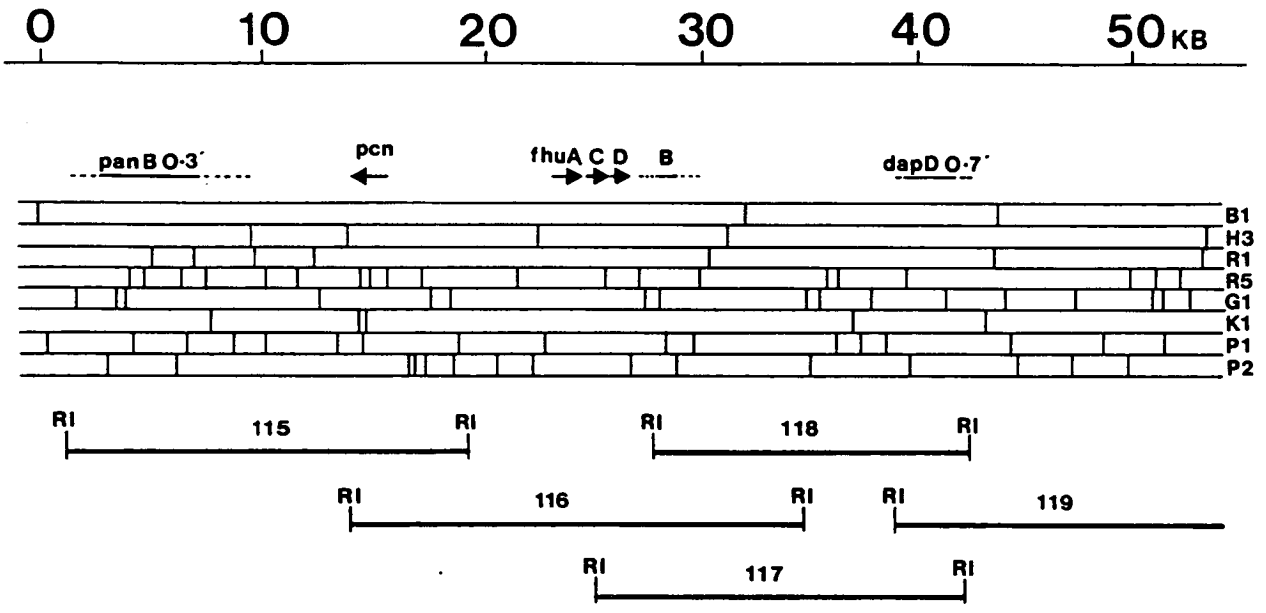
control. KH1331 pBR325 proved resistant up to 1200μg ml^{-1} of ampicillin, in contrast to JM18 pBR325, which could only grow up to 800μg ml^{-1}. The low ampicillin resistance of KH1331 pBR325 probably reflects the effect on DNA replication of the *dnaEts* mutation even at the permissive temperature, although it could be due to strain differences since JM18 and KH1331 are not isogenic. KH1331 pBR325 *pro*$^+$ transducants (100) were screened for temperature resistance; 8 had simultaneously become *dnaE*$^+$. All of these were able to grow on ampicillin plates at 1200μg ml^{-1}, indicating that *pcn* had not been transferred and therefore was not located between *dnaE* and *pro* (table 7.8b; figure 7.4). This indicated a map position for *pcn* between 3-4.5 minutes.

Co-transduction of *pcn* with *dapD* and *panB* was then attempted using AT986 pBR325 (*dapD*) and YA139 pBR325 (*panB*), by use of a P1Kc lysate made on JM18. Both AT986 pBR325 and YA139 pBR325 proved able to grow on high ampicillin, so they were transduced to either *dap*$^+$ or *pan*$^+$ and then screened for cotransduction of *pcn* (inability to grow on high ampicillin). Of the 117 YA139 pBR325 *pan*$^+$ transductants, 84 were now unable to grow on high ampicillin, indicating a cotransduction frequency between *panB* and *pcn* of 72%. This figure indicates a map distance of c.0.3 minutes between *panB* and *pcn* (Masters 1985). Of the 100 AT986 pBR325 *dap*$^+$ transductants, 37 were unable to grow on high ampicillin, indicating a cotransduction frequency of 37% and a corresponding map distance of c.0.7 minutes (Masters 1985). These results are shown in table 7.8b. From the mapping and transduction results it was concluded that *pcn* lay at 3.6 minutes on the *E.coli* genetic map, between *panB* and *dapD* (figure 7.4).

7.7 Location of *pcn* on the *E.coli* physical map

A precise localization of the *pcn* locus became possible after the publication of the entire restriction map of the *E.coli* chromosome, together with the availability of the corresponding λ clones covering the region in question (Kohara *et al.* 1987). Each λ clone contains about 20kb of insert DNA, and since the clones are overlapping it was possible to screen the entire region from 3-4 minutes. Clone numbers 115-120 were obtained, and each was tested for its ability to complement *pcn*. Since these λ clones are *cI*$^-$ *att*$^-$, in order to test for complementation it was first necessary to transform the recipient strain with the plasmid pcI^{857}. This plasmid carries the λ *cI*^{857} gene and kanamycin resistance together with a p15A replicon (M. Bagdasarian, pers comm. to M. Masters); thus in both its selection and replication it is compatible with

FIGURE 7.5 Location of *pcn* on the *E.coli* physical map



Restriction map re-drawn (with any additional restriction sites discovered during this study) from Kohara et al. 1987. **B1**= BamHI; **H3**= HindIII; **R1**= EcoRI; **R5**= EcoRV; **G1**= BglI; **K1**= KpnI; **P1**= PstI; **P2**= PvuII. Insert DNA contained within each of the λ clones is shown underneath. Note that insertion of the DNA into the vector introduces novel EcoRI sites at either end; thus the 5.5kb HindIII - EcoRI *pcn* fragment does not exist in vitro, but is the result of insertion into the vector.

pBR325. Although the λ clones are *att*$^-$, integration by homologous recombination at the appropriate chromosomal site should be possible.

To test for complementation of *pcn*, JM18 pBR325 pcI^{857} was grown to mid-log phase and a dilution was plated (in top agar) onto L-agar containing kanamycin (50μg ml^{-1}) and ampicillin (2000μg ml^{-1}). Several different dilutions were used in order to minimise background growth. An aliquot of each λ phage was spotted onto the surface of the agar and the plates incubated overnight at 30°C and 42°C. Clear complementation of *pcn* by clones 115 and 116 at 30°C was observed; a confluent area of growth around the phage spot being seen on the high ampicillin plate. At 42°C lysis was seen for all phage spots, demonstrating the integrity of the lysates.

Localisation of *pcn* on the physical map with respect to *panB* and *dapD* was then attempted. AT986 (*dapD*) was found to be complemented by clone numbers 118

and 119, while YA139 (*panB*) was complemented by clone 115. The locations of these clones, together with the mapped positions of *pcn*, *panB* and *dapD* are shown in figure 7.5. It should be noted that the relative cotransduction frequencies are in good agreement with the physical distances seen between markers.

7.8 Cloning of the *pcn* gene and visualisation of its protein product

TABLE 7.9 Transformation frequencies of *E.coli* chromosomal libraries into JM18 and MM18

STRAIN	LIBRARY	NO. OF TRANSFORMANTS LAamp50	LAamp1200
JM18	*Eco*RI	~2000	60
MM18	*Eco*RI	~2000	~2000
JM18	*Hin*dIII	~5000	40
MM18	*Hin*dIII	~2000	~2000
JM18	*Bam*HI	~5000	12
MM18	*Bam*HI	~5000	~5000

7.8.1 Screening of chromosomal libraries

Initially it was attempted to clone *pcn* by transforming JM18 with various *E.coli* chromosomal libraries cloned into pBR325, selecting directly for high ampicillin resistance. Three different libraries were screened; *Bam*HI and *Hin*dIII (M. Hanks), and *Eco*RI (H. Pringle). Transformants were plated on both high and low ampicillin plates. MM18 was transformed with all DNAs as a control. The resulting transformation frequencies are shown in table 7.9. While the number of MM18 transformants is the same on both high and low ampicillin plates, for JM18 the number of high ampicillin transformants is several hundred-fold lower than isolated on low ampicillin. This was observed for all three libraries. These 'high ampicillin resistant' clones were streaked to purity and their plasmids isolated and screened to determine insert size. However, although 12 clones from each library were screened, the insert size proved to be different in each case (data not shown). This suggested that either a large number of extragenetic suppressors of *pcn* exist, or the more likely explanation, that the cloned inserts were increasing ampicillin resistance by some means. The most plausible explanation appeared to be that promoters had been cloned into the vector which

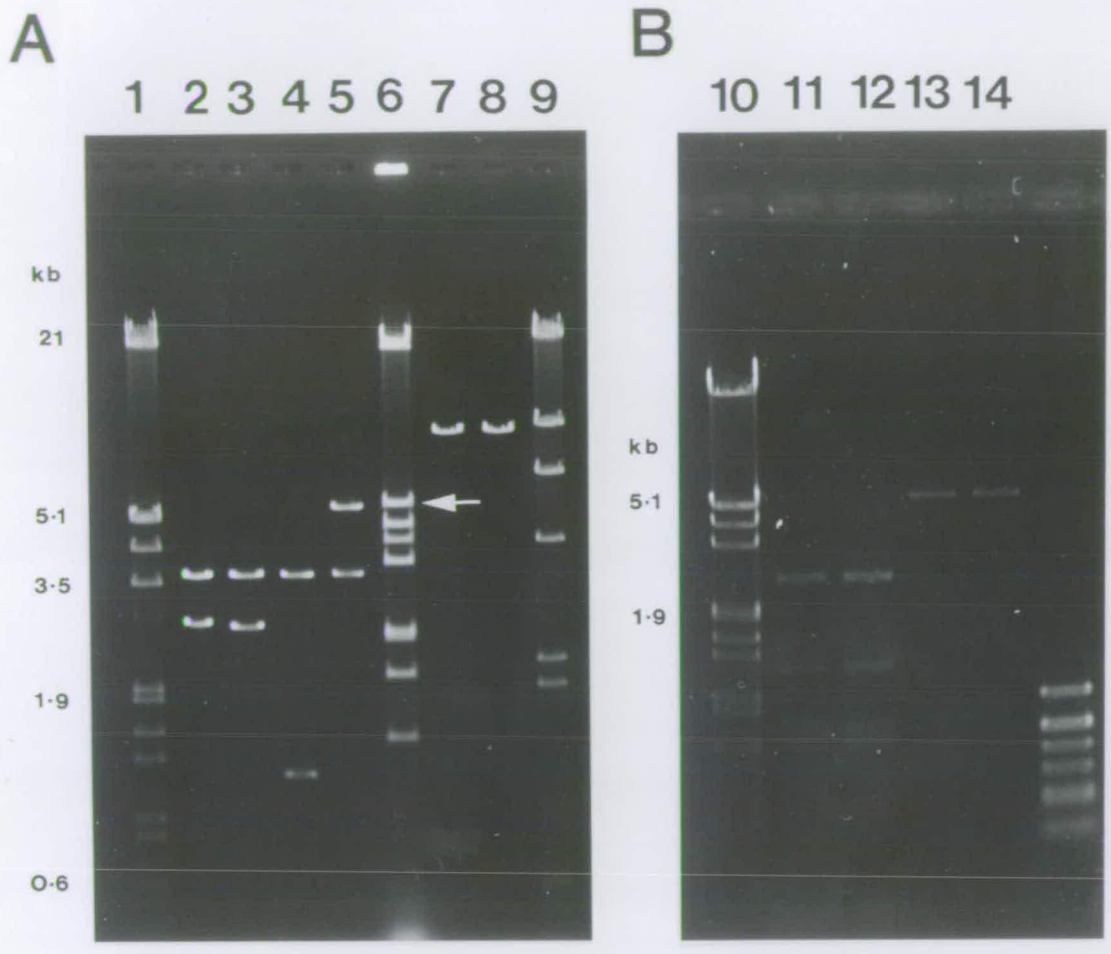
were reading into the β-lactamse gene and thus increasing its expression while not the actual copy number. This effect had previously been seen with the plasmid pHR3, which displayed high ampicillin resistance dispite having a lowered copy number in JM18 (section 7.2.5). Since there was no easy way of discovering which insert (if any) actually contained the *pcn* gene, it was decided to abandon this approach towards cloning *pcn*. Instead, it was decided to select for clones which were able to complement the *panB* mutation of YA139. These could then be screened for complementation of *pcn*. The relatively high cotransduction frequency between *pcn* and *panB* gave good grounds to hope that a concurrent cloning of both markers should be possible. However, this approach was abandoned when the λ clones described in the previous section became available, and it was decided to subclone the *pcn* gene out of one of these.

7.8.2 Cloning of *pcn* from λ115 clone: construction of pJM513

Since both λ115 and λ116 were able to complement *pcn*, this delimited the gene to the 5.3kb region common to both phages (figure 7.5). A feature of the construction of these phages is that the insert is bounded by *Eco*RI sites (Kohara *et al.* 1987); it was therefore decided to clone the 5.3kb *Hin*dIII-*Eco*RI fragment out of λ115 and into *Hin*dIII-*Eco*RI cut pBR328 (the *Eco*RI site being a feature of λ115 only; see figure 7.5). The *Eco*RI and *Hin*dIII restriction maps of both phages were checked and found to be accurate, the 5.5kb *Hin*dIII-*Eco*RI fragment of λ115 being easily visualised as the second largest fragment on an agarose gel (figure 7.6a).

To subclone the fragment, *Hin*dIII-*Eco*RI double digests of λ115 and pBR328 were run on an agarose gel and the appropriate fragments excised and eluted from the gel slice. The fragments were subsequently ligated and used to transform MM28-2 (a *recA* version of JM18), using pBR328 as a control. Transformants were selected on both high and low ampicillin plates. As expected, the pBR328 transformants grew on low ampicillin plates only. Use of the ligation mix resulted in transformants on both high and low ampicillin plates, although colony size in both instances was much reduced compared to the pBR328 controls (on low ampicillin plates). Transformants were then purified by streaking on both kinds of ampicillin plates and growth and colony size noted. As can be seen in table 7.10, although transformants isolated from the ligation mix are *pcn*$^+$ (MM28-2 pJM513), the presence of the insert fragment in high copy number causes a marked reduction in growth rate. The DNA from these transformants was purified and checked by restriction analysis. All showed the

FIGURE 7.6 Restriction analysis of *pcn* plasmids I



A Gel number 1. The 5.5kb HindIII-EcoRI *pcn* fragment of λ115 and pJM513 is marked with an arrowhead.

Track 1: λcI^{857} HindIII-EcoRI size standards
Track 2: pJM516 HindIII-EcoRI
Track 3: pJM511 HindIII-EcoRI
Track 4: pBR328 HindIII-EcoRI
Track 5: pJM513 HindIII-EcoRI
Track 6: λ115 HindIII-EcoRI
Track 7: pJM513 HindIII
Track 8: pJM513 EcoRI
Track 9: λcI^{857} HindIII size standards

B Gel number 2. Restriction of purified *pcn* fragment from above gel.

Track 10: λcI^{857} HindIII-EcoRI size standards
Track 11: λ115 HindIII-EcoRI *pcn* fragment
Track 12: pJM513 HindIII-EcoRI *pcn* fragment
Track 13: λ115 HindIII-EcoRI *pcn* fragment cut with PvuII
Track 14: pJM513 HindIII-EcoRI *pcn* fragment cut with PvuII

*Hin*dIII-*Eco*RI vector fragment together with the 5.3kb *Hin*dIII-*Eco*RI insert fragment (figure 7.6a. The DNA from λ115 was less pure than the plasmid DNA and thus ran slightly differently. As a result, the 5.3kb fragment appears to be slightly larger in λ115. To ensure that the correct fragment had been cloned, or that a small deletion had not occurred, both fragments were purified from this gel using GeneClean and further restricted before electrophoresis again. This is shown in figure 7.6b, and confirms that both fragments are indeed identical). The plasmid thus constructed was called pJM513 (λ115, *Eco*R1, *Hin*d3.

7.8.3 Subcloning of *pcn*: construction of pJM511 and pJM516

Following construction of pJM513, a more detailed restriction map of the 5.3kb insert fragment was prepared. This showed the presence of 4 *Pvu*II sites in the insert fragment rather than the two shown on the Kohara map. In addition, restriction mapping by Damon Hart-Davies has indicated the presence of 2 *Kpn*I sites in the insert fragment rather than the single site of Kohara *et al.* (figures 7.5 and 7.7). Essentially, all other sites examined were as per Kohara *et a.* (1987).

As several proteins are encoded by this insert fragment (section 7.8.5), further subcloning was necessary in order to determine which is the *pcn* gene product. To do this 'linker mutagenesis' was employed (see figure 7.8). Essentially, this involved performing a partial *Pvu*II digest on pJM513 and running the products out on a gel. The band corresponding to linear DNA could then be excised and purified from the agarose. This would give a collection of linear DNA molecules opened at different *Pvu*II sites. Either *Hin*dIII or *Eco*RI linkers could then be ligated onto the ends of these molecules. Two different approaches were then followed:

(1) The molecules were recircularised and transformed into MM28-2. If any of the *Pvu* II sites were within the *pcn* gene the introduction of the 8bp linker would alter the reading frame and result in the plasmid becoming *pcn*$^-$. The protein products of such a plasmid could then be analysed to see which had altered (and was thus the *pcn* gene product). Additionally, the location of this *Eco*RI or *Hin*dIII site could be easily mapped and thus provide a rough location for *pcn*. However, when this was done all the resultant transformants proved to be *pcn*$^+$, suggesting either a failure to ligate in the linkers or alternatively that *pcn* does not contain a *Pvu*II site (in fact the latter proved to be the case).

(2) The second approach was to digest such 'linkered' plasmid molecules with either *Hin*dIII (if *Hin*dIII linkers had been added) or *Eco*RI (if *Eco*RI linkers had been

FIGURE 7.7 Restriction maps of *pcn* plasmids

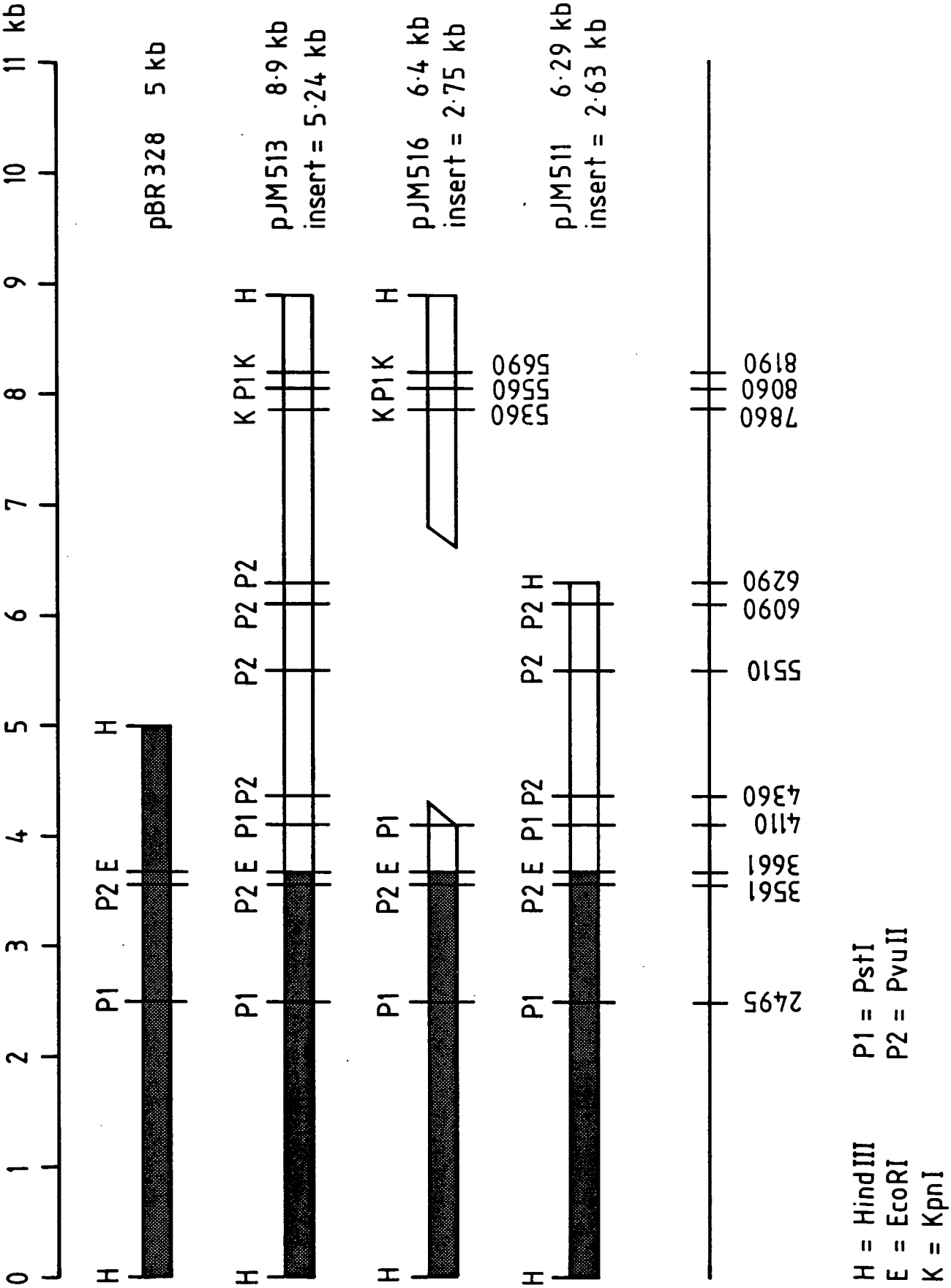
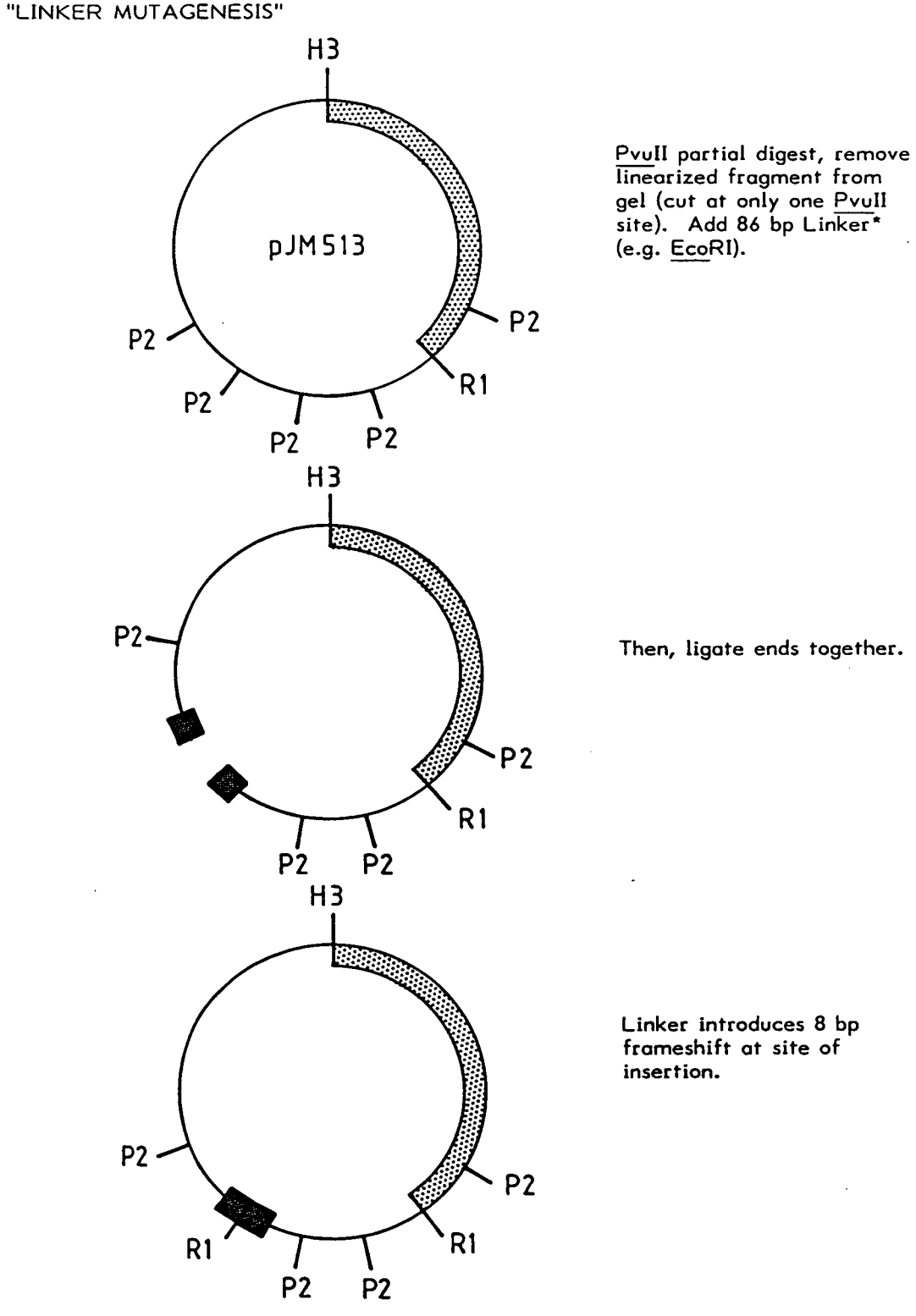
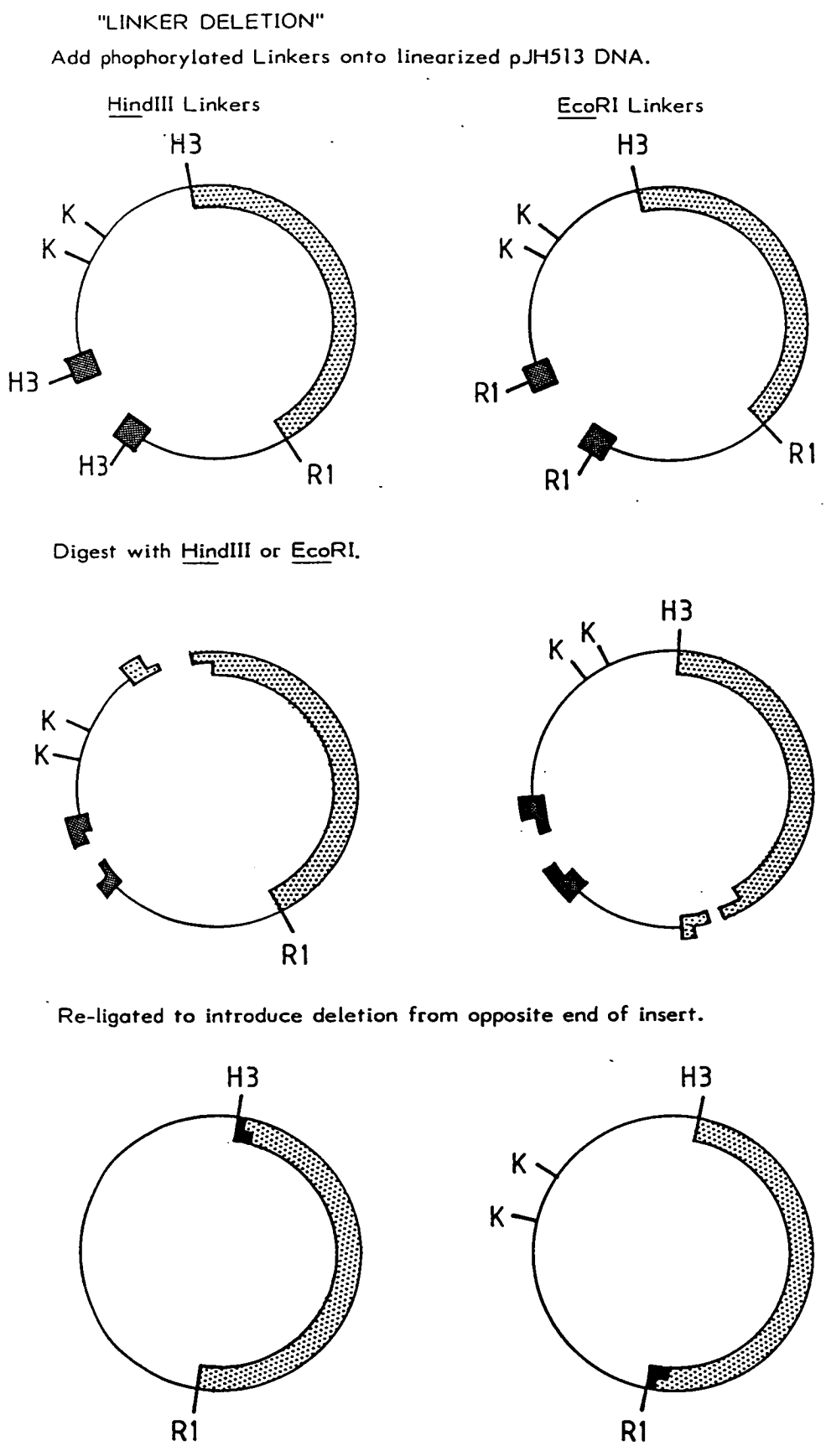


FIGURE 7.8 Linker mutangenesis of pJM513



* Use of unphosphorylated Linker ensures that only one strand of the linker is ligated on at each free end (therefore, multiple insertions cannot occur). Vector DNA shown dotted. H3 = HindIII sites, P2 = PvuII, R1 = EcoRI.

Figure 7.8: (continued)



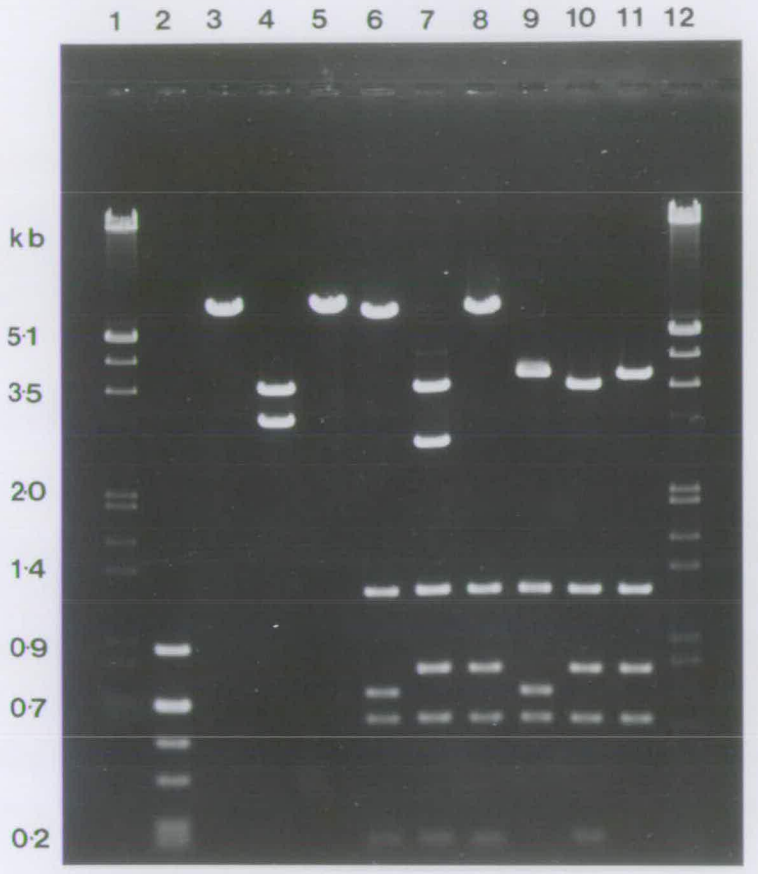
added). For the former case, this would result in the deletion of DNA between the *Hin*dIII site of pJM513 and the *Pvu*II site which had been opened and to which the linker had been added. Thus a series of deletions from the *Hin*dIII site to the different *Pvu*II sites could be isolated, which could then be screened for *pcn*$^-$. In the case of the *Eco*RI linkers, the deletions would occur from the *Eco*RI site; that is, from the opposite end of the insert fragment. This was done, and plasmids pJM511 (a *Hin*dIII deletion) and pJM516 (an *Eco*RI deletion) were isolated.

MM28-2 pJM511 is *pcn*$^-$, while MM28-2 pJM516 is *pcn*$^+$. Originally it was thought that these plasmids contained the opposite ends of the 5.3kb insert fragment, extending to the *Pvu*II site at position 6290bp of pJM513, with this site being changed to a *Hin*dIII site in pJM511 and to an *Eco*RI site in pJM516 (see figures 7.7 and 7.9). Essentially, pJM511 should have had the DNA between the *Pvu*II site at 6290bp and the *Hin*dIII site at 8900bp removed, and with pJM516 the DNA between the *Eco*RI site at 3661bp and the *Pvu*II site at 6290bp being removed. However, although this is true for pJM511, the deletion in pJM516 appears to be an aberrant event, not correlated with any of the *Pvu*II sites present. By further restriction mapping of pJM516 (figure 7.9 and data not shown) it proved possible to delimit the deletion fairly precisely. The *Pst*I site at 4110bp is present in this plasmid, while the *Pvu*II site at 4360bp is absent (figure 7.7). Therefore the end point of the 2.5kb deletion must lie within this region, the opposite end only being mapped to within 260bp also. However, this enabled localisation of the *pcn* gene to a 2.4kb stretch between the *Pvu*II (6.3kb) and *Hin*dIII (8.9kb) sites of pJM513.

7.8.4 Effect of *pcn* plasmids on growth rate

As has been mentioned above, pJM513 causes a marked reduction in growth rate when transformed into MM28-2. This effect is also seen when it is transformed into the minicell producing strain DS410, indicating that it is not due to the *pcn*$^-$ genotype of the host. Indeed, the growth rate of DS410 is so severely affected by this plasmid that it proved impossible to cultivate it without satellite colonies quickly overtaking the original transformant. This would appear to be the result of cloning the insert fragment in high copy number. An obvious question is whether over-expression of the *pcn* gene is responsible for this phenotype. To answer this, MM28-2 transformants of pBR328, pJM511, pJM513 and pJM516 were streaked out on L-agar containing ampicillin at 50μg ml^{-1} and the colony sizes compared. This is shown in table 7.10. While pJM516 does appear to cause a slight reduction in growth rate, it is

FIGURE 7.9 Restriction analysis of *pcn* plasmids II



Track 1: λcI^{857} HindIII-EcoRI size standards
Track 2: pBR322 AluI size standards
Track 3: pJM516 PvuII-EcoRI
Track 4: pJM516 PvuII-HindIII
Track 5: pJM516 PvuII
Track 6: pJM513 PvuII-EcoRI
Track 7: pJM513 PvuII-HindIII
Track 8: pJM513 PvuII
Track 9: pJM511 PvuII-EcoRI
Track 10: pJM511 PvuII-HindIII
Track 11: pJM511 PvuII
Track 14: λcI^{857} HindIII-EcoRI size standards

TABLE 7.10 Growth of MM28-2 *pcn* transformants

STRAIN	PLASMID	LAamp50	LAamp1200
COLONY DIAMETER (mm)			
MM28-2	pBR328	1.5	-
MM28-2	pJM513	<0.1	<0.1
MM28-2	pJM516	1.0	1.0
MM28-2	pJM511	1.5	-

Cultures were streaked to single colonies on high and low ampicillin plates and incubated overnight (c. 18hrs) at 37°C, whereupon colony diameter was measured.

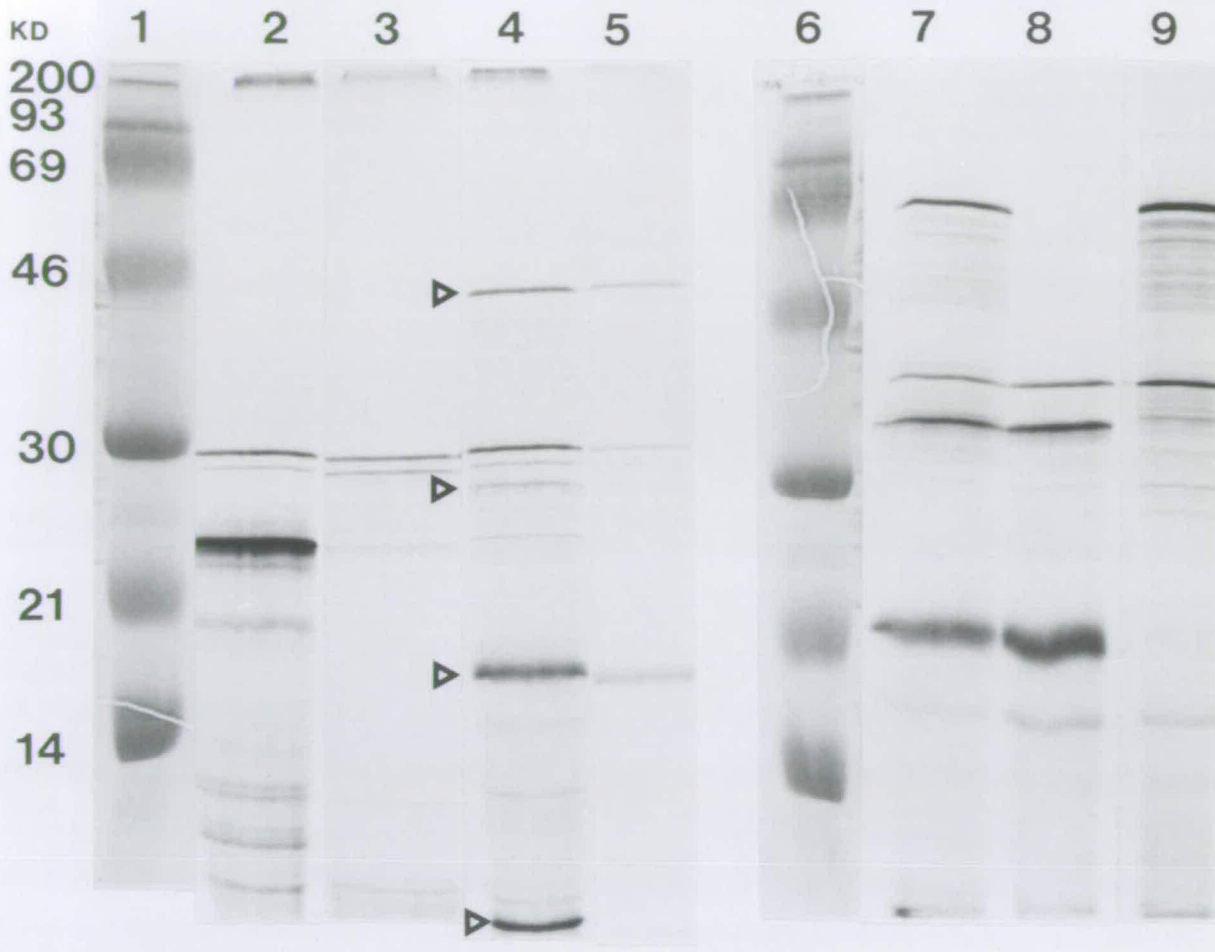
a small effect compared to that caused by pJM513. Both pJM511 and pBR328-containing strains display an identical colony size. Thus it appears unlikely that the effect on growth rate is due to over expression of the *pcn* gene product, although this cannot be totally discounted. It may be that more than one of the proteins encoded by the 5.3kb insert of pJM513 is required for the full reduction in growth rate observed with this plasmid, although the *pcn* gene product may be a contributory factor. Why pJM513 should cause a reduction in growth rate is unclear. MM28-2 pJM513 was examined under fluorescence to see if nucleoid segregation or DNA replication was affected compared to an MM28-2 control. However, no difference was observed between the cultures, with the degree of filamentation and segregation being apparently identical.

7.8.5 Visualisation of the *pcn* gene product

The probable identification of the *pcn* gene product was achieved using *in vitro* translation on pJM511, pJM513 and pJM516 purified template DNA. Initially it was hoped to identify the protein products of these plasmids using radiolabelling of minicell proteins as per pJM18 and pND5 in section 3.3. However, the severe reduction in growth rate caused by pJM513 effectively prevented such an approach and led to the adoption of the *in vitro* technique.

Prior to the construction of plasmids pJM511 and pJM516, the protein products of pJM513 were visualised using pBR328 as a control. Both undigested and *Hin*dIII-*Eco*RI digested template DNAs were used, in order to identify fusion

FIGURE 7.10 Proteins encoded by pJM511, pJM513 and pJM516



A Autoradiograph of *in vitro* translation number 1. The positions of the 48kd, 28kd, 17kd and 9kd protein bands unique to pJM513 are marked with arrowheads. 14-20% gradient gel.

Track 1: Molecular weight markers: Myosin, Phosphorylase b, Bovine Serum Albumin, Ovalbumin, Carbonic Anhydrase, Soybean Trypsin Inhibitor, Lysozyme

Track 2: pBR328

Track 3: pBR328 HindIII-EcoRI

Track 4: pJM513

Track 5: pJM513 HindIII-EcoRI

B Autoradiograph of *in vitro* translation number 2. The 9kd protein has run off the end of this particular gel. However, later gels showed it to be encoded by both pJM511 and pJM516, confirming that it is a fusion protein located at the EcoRI end of the insert fragment (data not shown).

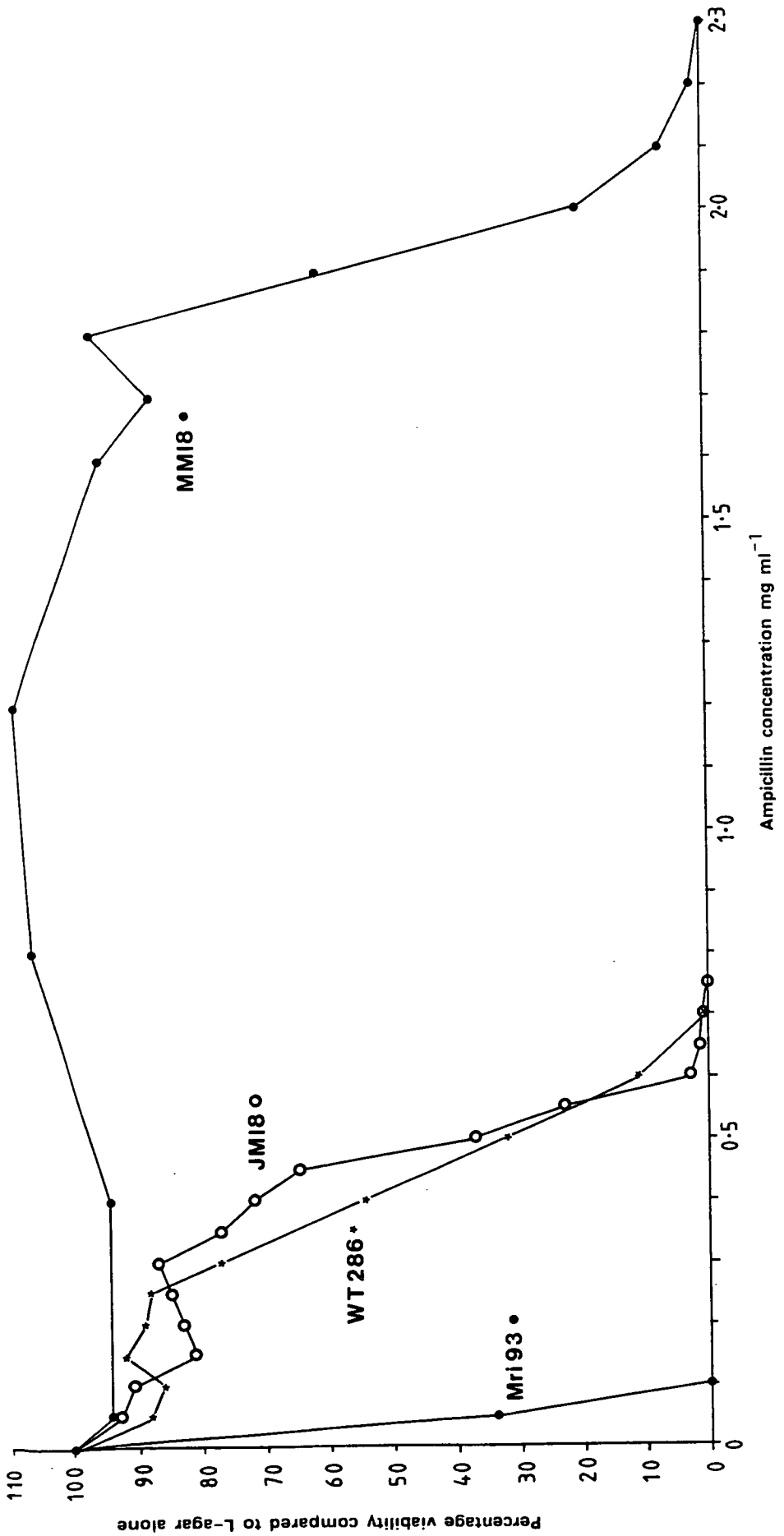
Track 6: Molecular weight markers as above

Track 7: pJM513

Track 8: pJM511

Track 9: pJM516

FIGURE 7.11 Ampicillin resistance of pBR325 in JM18, MM18 and Mri93



about 1800µg ml^{-1}) compares to a much lower level seen with the three *pcnB* strains. It would appear that pBR325 copy number in WT286 and JM18 is reduced to roughly the same extent, about 20-30% of that seen in JM18. The reduction in Mri93 is much greater however, a complete absence of single colonies being seen above 50µg ml^{-1} of ampicillin, suggesting that pBR-type plasmid copy number is not more than 1-2 in this strain.

That *pcnB93* causes a far more severe reduction in copy number than either *pcnB18* or *pcnB286* had already been suggested by observations made when transforming Mri93 pBR325 with pcI^{857} prior to complementation with the λ clones. Plasmid pcI^{857} uses the p15A origin of replication (Remaut *et al.* 1983; Chang and Cohen 1978); although this plasmid is compatible with ColEl replicons, its replication system is very similar and both plasmids share regions of homology at their replication origins. Additionally, both plasmids require the *polA* gene product (DNA polymerase I) for successful replication (Chang and Cohen 1978). Although stable maintenance of ColE1 and p15A is possible under normal conditions then, it seems likely that their replication systems are very similar. Thus replication of the p15A derivative pcI^{857} might also be affected in a *pcnB* strain.

Although double transformants of pBR325 and pcI^{857} were quite easy to isolate and maintain in both WT286 and JM18 (as might be expected following a relatively mild reduction in copy number), such a combination was almost impossible to maintain in Mri93. The number of colonies seen when a culture of Mri93 pBR325 pcI^{857} was plated under selection for both plasmids was reduced by a factor of 10^4 when compared to selection for individual plasmids. Thus two normally compatible plasmids were now behaving as if they were incompatible. If Mri93 can only maintain these plasmids at a copy number of 1-2 it is hardly suprising that the maintenance of 2 different plasmids which may be competing for the *pcnB* gene product is very unlikely. That pcI^{857} (p15A) is also dependent upon the *pcnB* gene product is further confirmed by its reduced antibiotic resistance in a *pcnB* strain (section 7.11.2). The dependence of other replicons upon the *pcnB* gene product is also discussed in this section.

Thus it would appear that low copy strains isolated from three independent laboratories are all mutant in the same gene, although the phenotypic effect of the mutation is somewhat different in each instance. The fact that all the low copy mutants studied seem to map to this locus would suggest a possible preference for this site. Since the *pcn* mutation appears to have no noticable effect upon the growth

rate of the host cell, this may explain the preference for copy number mutations to be located in this gene. It would be of some interest then to isolate a large number of copy number mutants to see if mutations mapping to another locus could be found or whether such a phenotype is the exclusive preserve of mutations in the *pcnB* gene.

7.10 Physiology of *pcn* strains

Perhaps the most noticable feature of the physiology of *pcnB* strains is the very absence of any change with respect to the parental. Apart from the reduction in copy number seen with some plasmids, no other detectable phenotype has yet been observed. Growth curves of JM18 and its isogenic pcn^+ control MM18 reveal no detectable difference. Similarly, the cell size distributions of JM18 and MM18 cultures show no difference. Thus no filamentation is occurring, as would be expected if host DNA replication were affected. Flow cytometry data (M. Masters, pers. comm.) further suggested that both the cell size distributions and DNA content of JM18 and MM18 are identical.

This would suggest that *pcnB* is not involved in *E.coli* DNA replication. Is it an essential *E.coli* gene though? The *pcnB18* mutation, while reducing the copy number of pBR-type replicons still obviously possesses some activity, since the effect is less severe than seen with *pcnB93*. Unfortunately, the parent of Mri93 was not available, so it was not possible to compare the effect on cellular physiology of this obviously more severe mutation in the *pcnB* gene. However, in the paper describing the isolation of this allele (Lopilato *et al.* 1986), no mention is made of an effect on growth rate in strains carrying this mutation, therefore it can probably be assumed that any effect is slight. A more accurate determination of the characteristics of the various *pcnB* alleles described in this section would probably be obtained if they were all transferred to the same background, allowing a more quantitative determination of copy number and effect on growth rate. The logical next step would then be to see if a null mutant of *pcnB* could be introduced into the *E.coli* chromosome and allow viability; this would reveal whether *pcnB* is an essential gene or not. The lack of a detectable phenotype for *pcnB* mutants would suggest that under normal laboratory conditions it is unlikely that *pcnB* would prove itself to be essential. Since DNA replication appears to be unaffected in *pcnB18*, this would tend to argue against the *pcnB* gene product having a role in chromosomal DNA replication.

7.11 Effect of *pcn* on replicons other than ColE1

An obvious question concerning the *pcnB* gene product is whether it is required by other replicons apart from ColE1. As was mentioned in section 7.9, the ColE1-like replicon p15A appears to be unstable in a *pcnB* strain, suggesting that it too depends upon the *pcnB* gene product for its replication. Is a requirement for *pcnB* restricted to ColE1-like replicons though, or is it required by a large range of plasmids for replication? Since DNA replication appears to be normal in a *pcnB* strain, this would suggest that *E.coli* DNA replication is not dependent upon the *pcnB* gene product. This was further tested by examining the transformation frequencies of a variety of different replicons into Mri93 and JM18 and its isogenic pcn^+ control MM18.

7.11.1 Transformation frequencies of various replicons into *pcn* strains

In order to investigate the effect of the *pcnB* mutation on transformation efficiencies of pBR-type plasmids, plus as a preliminary investigation into the effect of the mutation on other replicons, it was decided to compare the transformation frequencies of various plasmids into MM18, JM18 and Mri93. Although transformation frequencies obtained with Mri93 are not strictly comparable to the isogenic *pcnB18:pcnB*$^+$ strains JM18 and MM18, it was included by virtue of the more severe effect on copy number of the *pcnB93* allele in this strain. It was considered that some low copy plasmids might require the *pcnB* gene product and yet not show a demonstrable difference in transformation frequency between JM18 and MM18 due to the relatively mild reduction in copy number in the former. Therefore it was hoped that use of Mri93, with its more severe effect on pBR-type plasmid copy number might increase any differences in transformation efficiency seen between *pcnB* and *pcnB*$^+$ strains.

The different plasmids used, their antibiotic resistances, and the resulting transformation frequencies are shown in table 7.11. It is apparent from this data that the transformation frequencies of JM18 and MM18 are very similar for all plasmids tested, reflecting perhaps their isogenic backgrounds. In addition however, this indicates that an examination of transformation frequencies is too crude a test of the dependence of a particular replicon upon the *pcnB* gene product. Plasmid pBR328 for example, although it has a copy number in JM18 of only 25% of that in MM18, shows no difference in transformation efficiency. Therefore it is not possible from transformation data alone (at least on low antibiotic concentrations) to determine whether a particular replicon requires the *pcnB* gene product. A comparison of these

TABLE 7.11 Transformation frequencies of *pcn* strains with different plasmid replicons

PLASMID	REPLICON	ANTIBIOTIC	REDUCTION[a] RESISTANCE	Mri93	JM18	MM18
Transformants per µg of DNA						
BR328	*pMB1*	Amp	2×10^4	2.1×10^2	4.1×10^6	3.9×10^6
pJM516	*pMB1*	Amp	108	3.7×10^5	4×10^2	4×10^7
pHP6	*oriC*	Chl	32	1.9×10^6	6×10^7	6×10^7
pcI^{857}	*p15A*	Kan	70	9.5×10^4	6.4×10^6	6.1×10^6
pVH1	*ColD*	Kan	40	7.8×10^4	3.4×10^6	3.1×10^6
pCB106	λ *dv*	Chl	58	1.2×10^6	7×10^7	7×10^7
pPM30	*pSC101*	Amp	74	9.4×10^5	7×10^7	7×10^7
pKN500	*RI*	Kan	180	2.5×10^4	2×10^6	4.5×10^6
pGW71	*RI*	Amp	100	4.1×10^5	1.2×10^7	4×10^7

a. Reduction of transformation efficiency in Mri93 compared to MM18

results with the transformation frequencies obtained with Mri93 is difficult due to the generally reduced transformation efficiency obtained with this strain. Comparison with MM18, for all plasmids with the exception of pBR328 reveals a general reduction of between 10^1–10^2 in transformation frequency. The only plasmid where a significant difference in transformation efficiency is seen is pBR328, which suffers a drop in transformation frequency of 10^4 compared to MM18, although as noted above, this reduction is not seen in the other *pcnB* strain JM18. In fact, this reduction is probably due as much to the very low ampicillin resistance conferred by pBR-type plasmids in this strain as much as it is due to a lowered transformation efficiency (as can be seen in figure 7.11, Mri93 pBR325 is unable to grow above an ampicillin concentration of

50µg ml^{-1} when present as well-isolated single colonies, such as would occur during a transformation experiment for example). This is further suggested by the results obtained with plasmid pcI^{857}, which has the ColE1-type replicon p15A, and which earlier data had suggested was affected by *pcnB* (section 7.9). No specific reduction in transformation frequency is seen with this plasmid, which carries kanamycin resistance rather than the ampicillin resistance of pBR328.

Thus, examination of the transformation frequencies obtained with $pcnB^-$ and $pcnB^+$ strains reveals no significant differences between them which might be used as a means of determining whether or not a particular replicon requires the *pcnB* gene product.

7.11.2 A comparison of the antibiotic resistances of various plasmids in Mri93, JM18 and MM18

Due to the failure of the transformation frequency data as a means of determining a requirement for the *pcnB* gene product, it was decided to estimate the relative copy numbers of the above plasmids in each background by means of the level of antibiotic resistance conferred. Unfortunately, the plasmids carried a variety of different antibiotic resistances; ampicillin (pPM30 and pGW71), chloramphenicol and tetracycline (pHP6), and kanamycin (pcI^{857}, pVH1 and pKN500). Therefore a detectable difference would require that resistance to each antibiotic be at least partially proportional to the gene dosage of the resistance gene. Stationary phase liquid cultures of each of the three strains containing the various plasmids to be tested were diluted down by a factor of 10^4, and 50µl plated onto varying concentrations of the antibiotic. The plates were incubated overnight at 37°C and the number of colonies on each concentration of antibiotic counted. If too large a number of colonies were present to enable a realistic count of the entire plate, then an estimate was made by counting a proportion of the plate and multiplying accordingly. The results of this experiment are plotted graphically as the percentage survival at any given antibiotic concentration (compared to the number of colonies present on L-agar alone = 100%) in figure 7.12.

The resistance figures for pMB1 replicons have already been presented elsewhere (section 7.9 and figure 7.11). The resistance figures for plasmid pHP6 (*oriC*) on both chloramphenicol and tetracycline plates reveal no detectable differences between any strain, suggesting that this plasmid does not require the *pcnB* gene product. However, it should be remembered that resistance to chloramphenicol is not

FIGURE 7.12 Antibiotic resistance curves of various plasmids in JM18, MM18 and Mri93

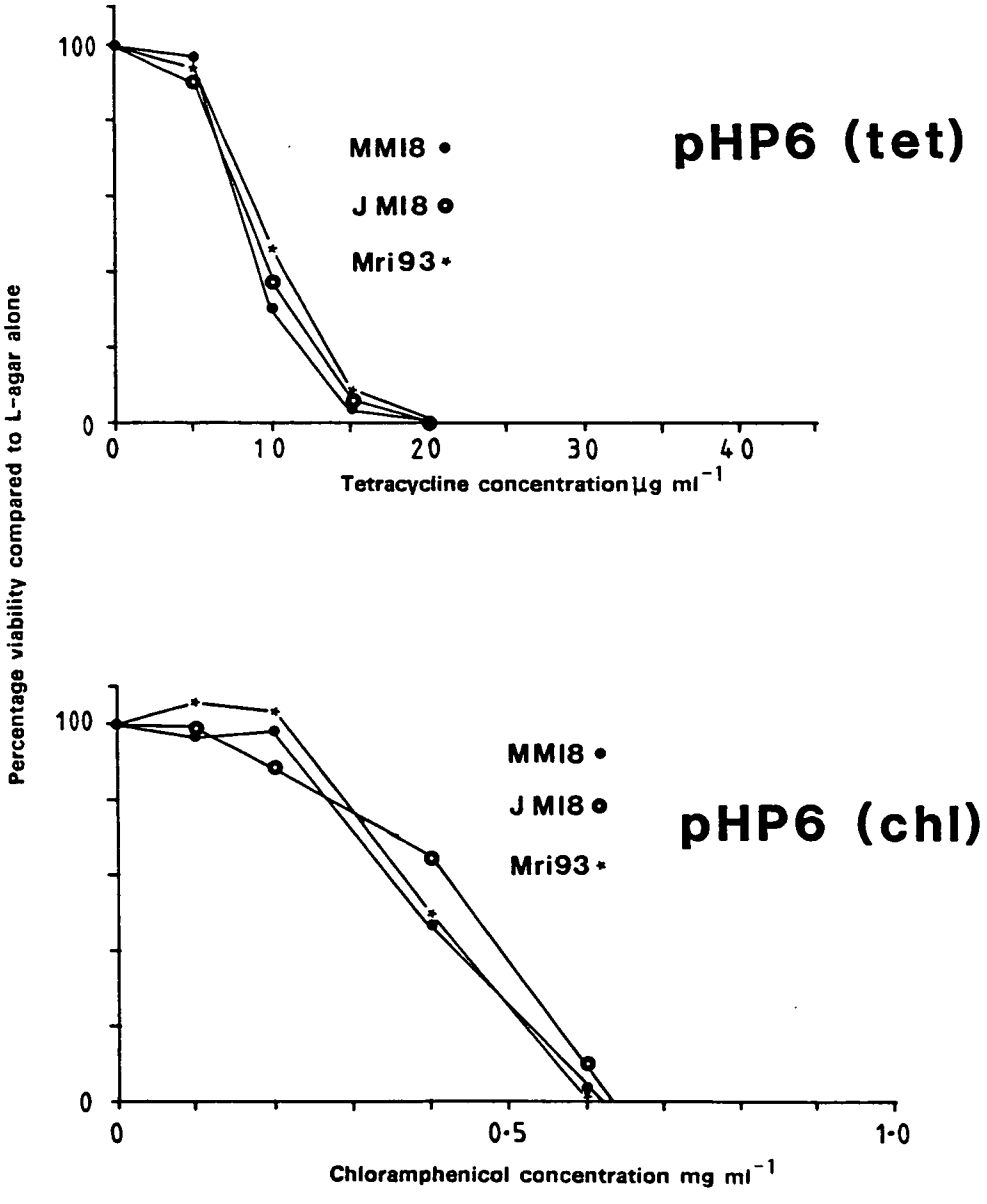


Figure 7.12: (continued)

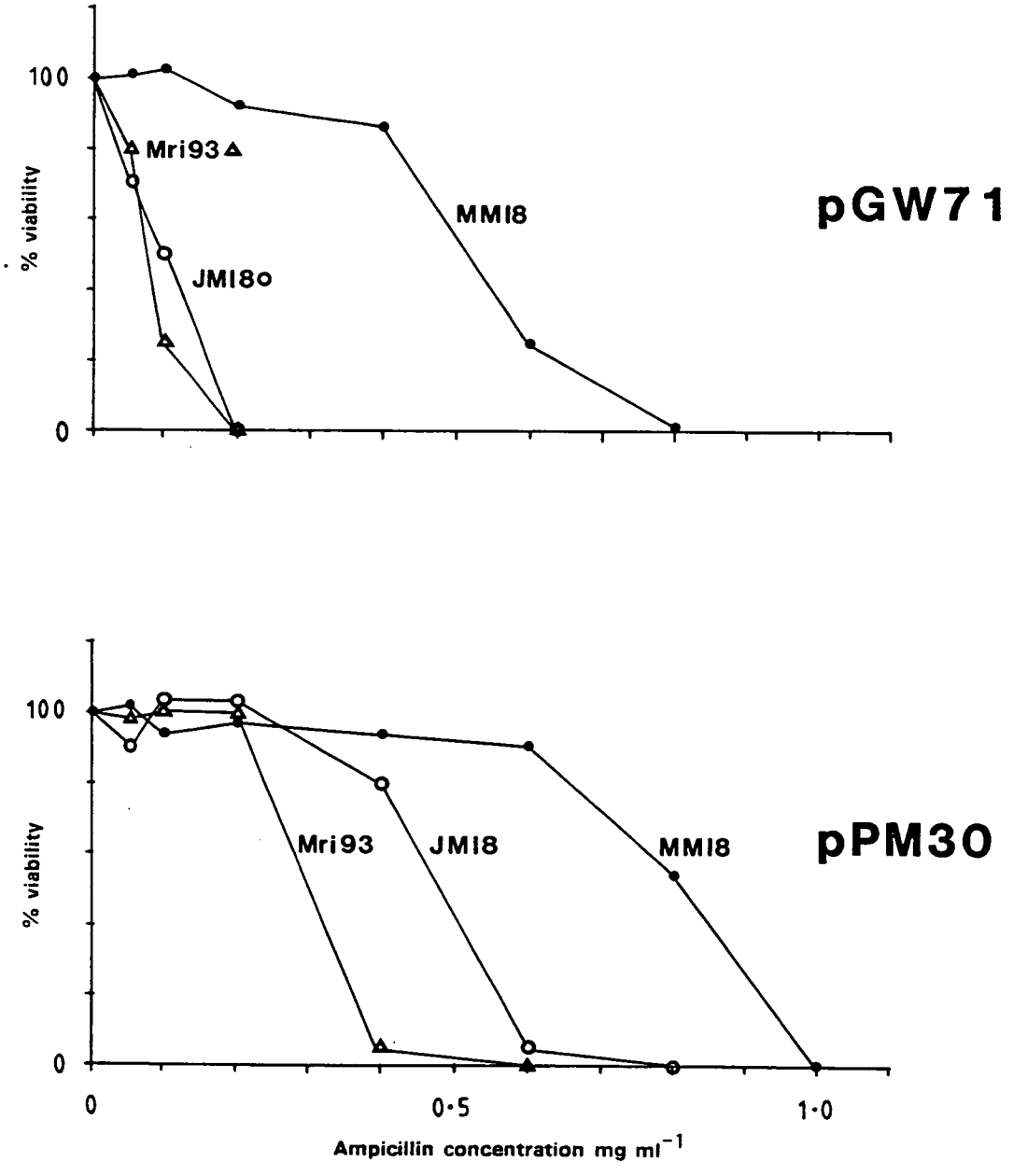
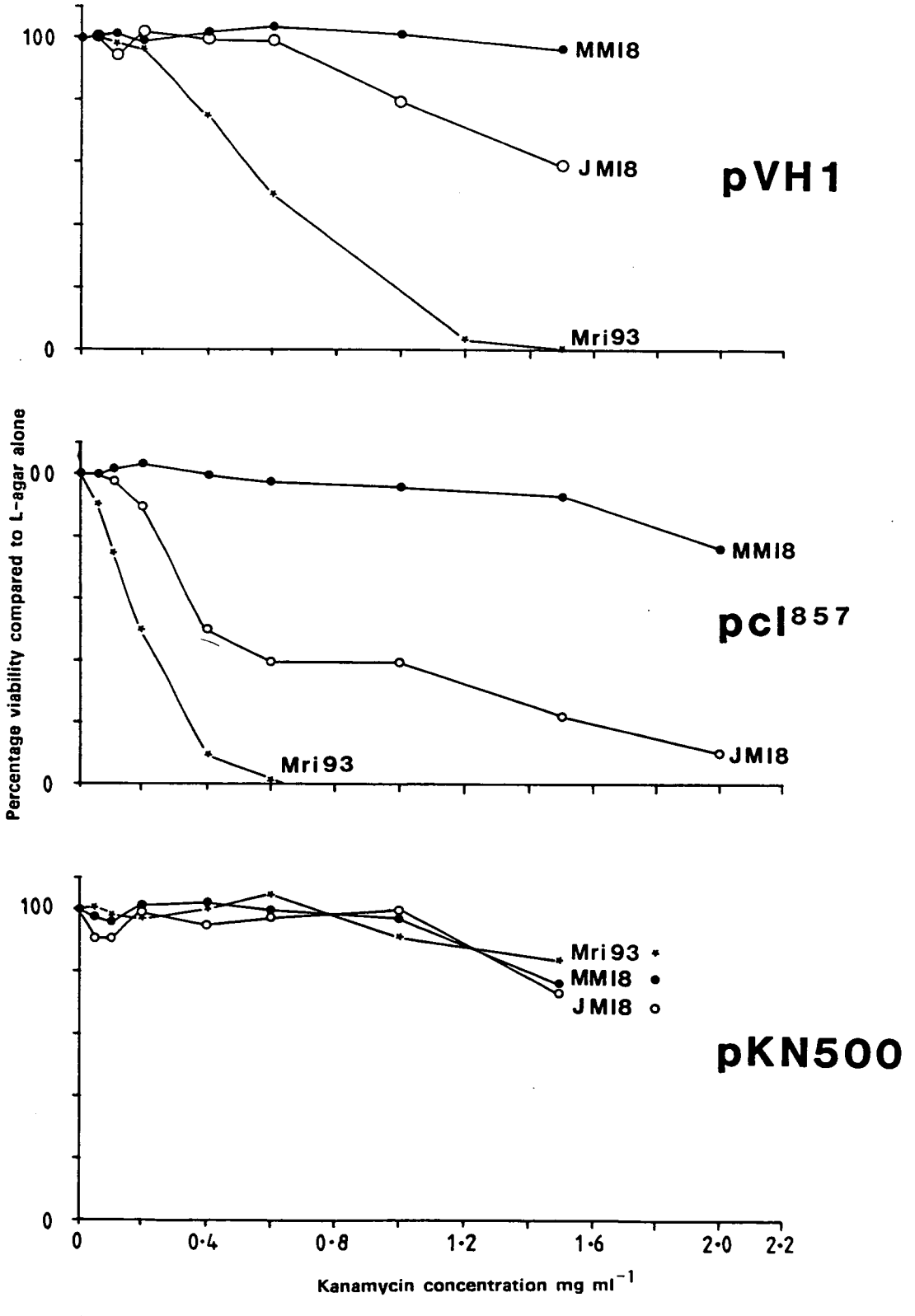


Figure 7.12: (continued)



particularly linear with respect to gene dosage (section 7.5), so it is possible that differences in the copy number of the plasmids between strains would not be detected using this approach. However, the figures obtained for tetracycline resistance, which is more linear with respect to gene dosage (section 7.5) also reveal no difference, suggesting that any effect of *pcn* on pHP6 must be small and outwith of the resolution of this system. (At a later date, curing curves performed on MM18 pHP6 and JM18 pHP6 revealed no difference in the rate of plasmid loss, confirming that pHP6 does not have a lowered copy number in JM18; Ian Oliver pers. comm.).

The ampicillin resistance curves obtained for the plasmids pPM30 (pSC101) and pGW71 (mini-R1) reveal a reduced resistance in both *pcn* strains, suggesting that both these replicons are at least partially dependent upon the *pcnB* gene product. Plasmid pPM30 would appear to have a generally higher copy number than pGW71 from these resistance figures, although interestingly, the reduction in ampicillin resistance between *pcn*$^+$ and *pcn*$^-$ strains appears much less pronouced with these plasmids than with pBR325 (section 7.9 and figure 7.11). For example, Mri93 pBR325 is resistant up to amp50, while for MM18 pBR325 the figure is amp2000. For pPM30 the figures are amp200 and amp600 respectively, suggesting that although pPM30 originally has a lower copy number than pBR325, the reduction in a *pcn*$^-$ background is much less pronounced, perhaps due to a reduced dependence on the *pcnB* gene product for this replicon.

The kanamycin resistance curves suggest a reduced copy number for both pVH1 (ColD) and pcI^{857} (p15A) in a *pcn* background. For plasmid pcI^{857} this agrees with the instability noted for this replicon in a *pcn* background (section 7.9). In addition, the reduction in antibiotic resistance in Mri93 appears to be more severe than that observed with JM18. For plasmid pKN500 (mini-R1), the kanamycin resistance curves suggest this plasmid to be unaffected by the *pcn* mutation. This result appears contradictory to that obtained with pGW71 (also mini-R1) which did appear to have a reduced copy number in a *pcn* strain. Curing curves performed on these plasmids revealed that the stability of both of them is lowered in JM18, suggesting a dependence on *pcn*. It is unclear why a reduced kanamycin resistance is not observed with pKN500 in JM18. One possibility is that kanamycin resistance is not able to detect the difference between the copy number of this plasmid in a *pcn*$^-$ and *pcn*$^+$ strain. No information is available at present regarding the linearity of kanamycin resistance to gene dosage. Therefore it is possible that kanamycin resistance, like chloramphenicol resistance, is not particularly proportional to gene dosage. However, the kanamycin resistance curves obtained with pVH1 and pcI^{857} suggest that this is

probably not the case. For a more definitive examination of the effect of the *pcn* mutation on R1 plasmids, the measurement of copy number by quantitative hybridisation should be performed.

7.11.3 Stability of F replicon in *pcnB* strain

To investigate whether the *pcnB* gene product is required for stable maintainance of the F replicon, it was decided to investigate the stability of an F-Prime in the absence of selection in a *pcnB* strain. Since F is maintained in a copy number of 1, obviously there is no purpose in examining whether F has a lowered copy number in a *pcnB* strain. If F replication is affected in such a strain it should be less stable, and subject to loss in the absence of selection. Accordingly, strain E11022 F'*his* was mated into both MM18 and JM18 by selecting for growth on *his*$^-$ medium. Clones were purified and then grown in liquid medium in the absence of selection for *his*$^+$, followed by dilution down to single colonies. These were patched onto *his*$^-$ and *his*$^+$ media to see if any loss of the F-Prime had occurred. Of the 200 MM18 and JM18 F'*his* colonies tested, non had become *his*$^-$, indicating that F is stable in a *pcnB* strain, and suggesting that it does not require the *pcnB* gene product. A problem with this approach is that the F-Prime could be continually transferred by mating into strains which had lost it during growth. However, since the culture was grown up with vigorous shaking this risk was assumed to be small. Additionally, it should be remembered that since F is maintained in a unit copy, factors which affect segregation rather than replication might play a more important role in the stability of this plasmid. It is possible that F requires the *pcnB* gene product, but its low copy number means that the mutant phenotype is not apparent since its requirement for the *pcnB* gene product might be reduced accordingly.

7.11.4 Lytic DNA replication of phages T7, P1 and λ in a *pcnB* strain

To investigate the effect of the *pcnB* mutation on the lytic DNA replication of phages T7, P1 and λ, a lysate of each was titred on JM18 and MM18, and the titre and plaque size compared. A P1kc lysate, a T7 wild type lysate and a λ *vir* lysate were all tested, and both the plaque size and titre of the lysates proved to be the same on both JM18 and MM18. This suggested that the *pcnB* gene product is not involved in the lytic DNA replication of either of these phages, although the possibility that it is involved in the replication of P1 as a lysogenic plasmid cannot be discounted.

7.11.5 Summary

Several replicons in addition to pMB1 appear to be affected by *pcn*. These include p15A, ColD, pSC101 and R1. The large conjugative plasmid F appears to be unaffected in a *pcn* strain, as does an *oriC* plasmid. The replication of phages λ, P1 and T7 appears to be unaffected by *pcnB* also.

A more accurate examination of the effect of *pcnB* on the various plasmid replicons would probably involve performing curing curves and directly measuring plasmid copy number by quantitative hybridisation. Although antibiotic resistances can give some indication of the copy number of a particular replicon, this is only an estimate. The use of such measures should give a definitive answer regarding the requirement of a particular replicon for the *pcnB* gene product.

7.12 Isolation of other low copy mutants

The *pcnB* mutant strain JM21 was originally isolated by virtue of the fact that over-expression of the *groE* genes causes a deleterious effect on cell growth in conjunction with certain *dnaAts* alleles at 30°C. This cold sensitive phenotype was due to the cloning of the *groE* genes on a high copy plasmid, in this instance a pMB1 derivative (pBR325). Thus it should be possible to isolate a large number of potential copy number mutants by the same approach: namely by looking for colonies which have simultaneously lost their cold sensitivity and the ability to grow at 42°C. These can then be screened to see if this is a result of a reduction in over-expression of *groE* due to a lower plasmid copy number. These potential copy number mutants can then be screened, and if the above phenotype is indeed due to a lower plasmid copy number, the causative mutation can then be mapped. It may be possible to use this approach to isolate copy number mutations which affect plasmids other than ColE1-types, by cloning the *groE* genes (or other genes which are deleterious in high copy) into replication origins other than those affected by *pcnB*. However, such mutants may also affect host cell DNA replication, thus effectively preventing their isolation. A feature of such an approach is that it attempts to isolate mutants which affect plasmid replication without causing ill-effects to the host; for most plasmids the two demands might not be compatible. However, it may be possible to identify the genes encoding the primosomal proteins n, n' and n" (Kornberg 1980) by use of such an approach.

TABLE 7.12 Stability of pND5 in MM182 background

ISOLATE	LA	LAamp50	PLASMID LOSS
NUMBER OF COLONIES			
JM182-1 pND5	45	29	36%
JM182-2 pND5	100	100	0%
JM182-3 pND5	100	100	0%
JM182-4 pND5	100	100	0%
JM182-5 pND5	100	80	20%
JM182-6 pND5	100	100	0%
JM182-7 pND5	80	80	0%
JM182-8 pND5	100	78	22%
JM182-9 pND5	50	45	10%

Single colonies of MM182 pND5 (non-cold sensitive and unable to grow at 40°C) were patched onto LA and LAamp plates and incubated overnight to look for plasmid loss.

To test the feasibility of such an approach, it was decided to try and isolate further low copy mutants by using the same technique that yielded JM21. To do this, MM182 (*dnaA5ts*) pND5, and MM185 (*dnaA46ts*) pND5 were streaked out at 42°C and single colonies isolated. Growth at 42°C maintained selection for pND5, such that all colonies should be normal high copy type. JM21 was isolated as a derivative of MM185 pND5; MM182 pND5 was also included since the cold sensitivity caused by *groE* over-expression in conjunction with this allele is much more severe, and thus should exert a stronger selective force against a high plasmid copy number. Ten single colonies of each type were transferred to 5ml of L-broth containing ampicillin at 50μg ml^{-1} (to ensure that plasmid free colonies were not simply isolated), and grown to stationary phase under conditions of cold sensitivity (growth at 30°C). The cultures were then streaked out at 30°C on L-agar containing ampicillin at 50μg ml^{-1}, and incubated overnight at 30°C. Large (non cold sensitive) colonies were seen on each plate. These were then patched at 30°C and 42°C to see if any had lost the ability to grow at the latter temperature (potential low copy mutants). MM182 pND5 and MM185 pND5 strains were included as controls. Of the MM185 pND5 colonies, all were able to grow at 42°C, indicating that the loss of cold sensitivity was not due to a reduction in the amount of GroE present. These were discarded as probably not being copy

number mutants. Of the 10 MM182 pND5 colonies, 9 were now unable to grow at 42°C. A single colony of each of the 9 was then transferred to 5mls of L-broth (no selection) and grown at 30°C to test for curing of the plasmid. Once the culture had reached stationary phase it was diluted down to single colonies, and these were patched and tested for plasmid loss. The results are shown in table 7.12. Of the 9 potential copy number mutants, 5 exhibited no plasmid loss, suggesting that the observed phenotype is probably due to a mutation which reduces the expression of *groE* somehow. However, 4 of the strains displayed a higher level of plasmid loss, ranging from 10% to 36%. These strains are most probably copy number mutants, although no further investigation of them was undertaken. They were called JM182-1, JM182-5, JM182-8 and JM182-9, and they demonstrate that use of such an approach to isolate low copy mutations is a repeatable phenomena.

7.13 Discussion

The results presented in this chapter describe the isolation of an *E.coli* chromosomal mutation, *pcnB*, originally observed as causing a reduction in the copy number of pBR-type plasmids and their derivatives. Further study has indicated that pSC101 and a mini-R1 plasmid are also affected in a *pcnB* strain, while F, and *oriC* plasmids appear to be unaffected. It is unclear at present whether a larger R1 derivative requires the *pcnB* gene product. Lytic DNA replication of bacteriophages T7, P1 and λ appears to be unaffected in a *pcn* strain.

The *pcn* mutation described in this study has been shown to be allelic to three other, independently isolated low copy mutants. The reduction in pBR-type plasmid copy number in the *pcnB* mutant described in this work (tentatively entitled *pcnB18*) is to a level 20-25% of that seen in the wild-type parental, as measured both by antibiotic resistance levels and quantitative hybridisation. The reduction in plasmid copy number appears to vary depending on the allele. However, no effect on host cell metabolism as a result of this mutation has been observed (either in DNA replication or growth rate).

Using a mixture of Hfr mapping, P1 transduction, and direct complementation using overlapping λ clones, the *pcnB* gene has been mapped to 3.6 minutes on the *E.coli* genetic map, between the *panB* and *dapD* genes. Complementation studies have indicated that *pcnB18* is recessive in nature. The gene has been cloned on a high-copy vector, and a 48kd protein has been identified as likely to be the *pcn* gene product.

How might this chromosomal mutation result in a reduced plasmid copy number, and additionally, what features determine the observed specificity regarding which plasmids are affected? Furthermore, is *pcnB* a newly identified gene or is it allelic to a previously identified genetic marker?

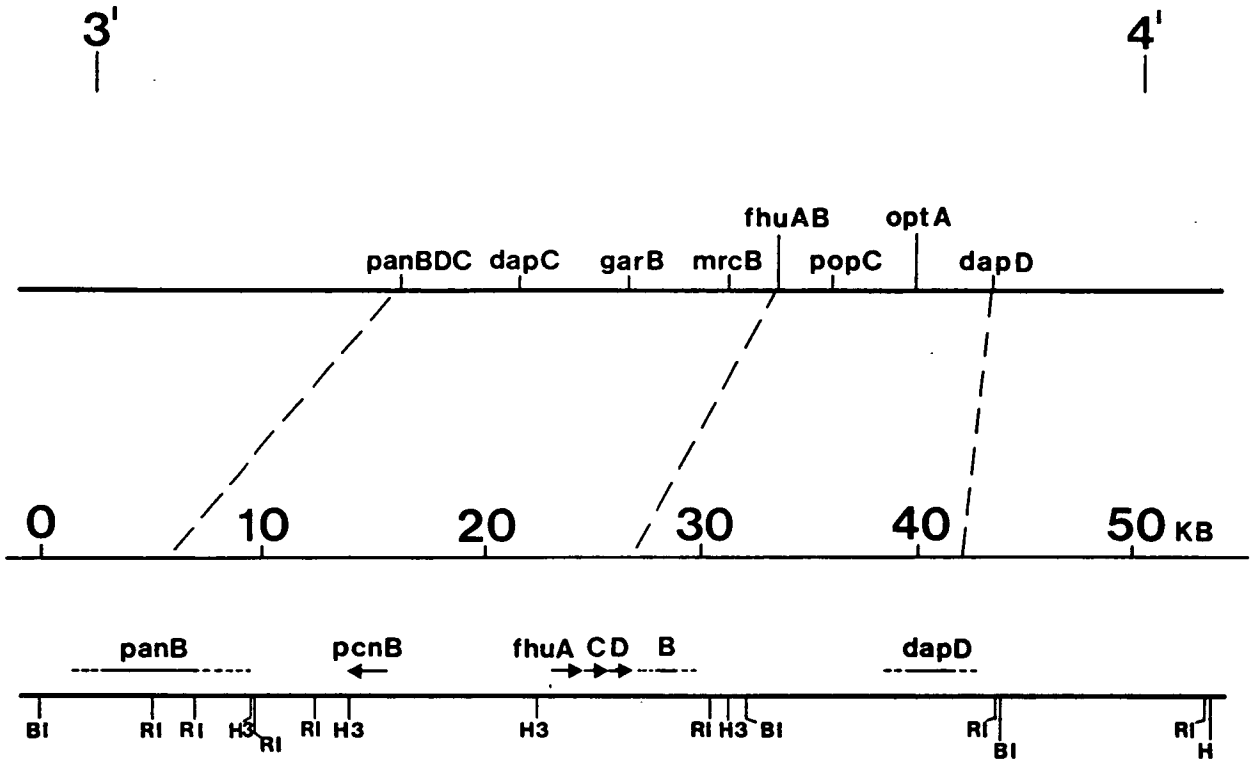
I shall first consider the second question by making reference to the genetic markers thought to be located between *panB* and *dapD*. I shall then discuss possible modes of action for the *pcnB* gene product by making a detailed survey of the replication control systems of a variety of plasmid replicons, both Pcn-requiring and Pcn-independent, in the hope that some common features may become apparent.

7.13.1 Is *pcnB* a newly-identified gene?

Examination of the *E.coli* genetic map covering the region from *panB* to *dapD* (Bachmann 1983) reveals the presence of 6 distinct genetic loci within this intervening space (figure 7.13). These loci are (reading from *panB* to *dapD*), *dapC, garB, mrcB, fhuAB, popC* and *optA*. Might *pcnB* be allelic to any of these genetic markers?

Beginning with *dapC*, I have been informed (Catherine Richaud pers comm.) that such an marker does not in fact exist at 3.7 minutes on the *E.coli* genetic map, and that the *dapC* mutant originally isolated by Bukhari and Taylor (1971) is actually a *dapA* mutant located at 53 minutes. The next genetic marker is *garB*, which encodes a product involved in glucarate utilisation, mutants in this gene being defective in both the transport and metabolism of this carbohydrate (Robertson *et al.* 1980). However, this gene is unlikely to be allelic to *pcnB* for two reasons. Firstly, it is difficult to conceive of how an outer membrane protein concerned with metabolite transport can be involved in the control of plasmid copy number. Secondly, and perhaps more significantly, *garB* was measured as being 96% cotransducible with *dapD*, a far higher figure than was measured for *pcnB* (37%). This would suggest that *garB* is not allelic to *pcnB*, and that it is located much closer to *dapD* than is drawn on the *E.coli* genetic map. Additionally, Robertson *et al.* (1980) reported that they were unable to separate this marker from the adjacent *fhuAB* locus, suggesting that these two loci were probably allelic. This is further suggested by the function of the *fhuAB* (alternatively known as *tonA*) gene product, which is an outer membrane protein involved in ferric uptake. In addition, it is the receptor molecule for coliphage T5, and acts as the colicin M receptor protein (Braun *et al.* 1973). Thus it is quite conceivable that the *garB* mutant of Robertson *et al.* is simply an allele of *fhuAB*, given the similarity of function and cellular location. Since the 1983 edition of the *E.coli* genetic

FIGURE 7.13 Genetic and physical map of the *pcn* region of the *E.coli* chromosome



Map based on cotransduction data (Bachman 1983) shown uppermost. Distances shown in minutes. Below is shown the physical map, using data from Kohara et al. (1987), Fecker and Braun (1983) and this study.

map (Bachmann), it has become apparent that the *fhuAB/tonA* marker in fact comprises four distinct genetic loci, ordered *fhuA-fhuC-fhuD-fhuB*. The region has been cloned, and the protein products of the first three genes have been visualised (Fecker and Braun 1983). The genes encode proteins of 78kd (fhuA), 30kd (fhuC) and 26kd (fhuD) (Kadner *et al.* 1980, Fecker and Braun 1983). The *fhuB* gene product has not been visualised. Notwithstanding that none of these protein products are of the correct size for the presumed *pcnB* gene product, examination of the restriction map of the clones constructed by Fecker and Braun has allowed direct localisation of these genes on the *E.coli* physical map of Kohara *et al.* (1987). This is shown in figure 7.13, and demonstrates that the *fhuACDB* region is located between *pcnB* and *dapD*. Might *pcnB* be allelic to *mrcB* (alternatively called *ponB*)? The gene product of *mrcB* is Penicillin Binding Protein 1b, involved in peptidoglycan biosynthesis, and a major target

of the β-lactam group of antibiotics (Tamura *et al.* 1980). Again, the function and location of the *mrcB* gene product would tend to argue against it being allelic to *pcnB*, and additionally, the purification and identification of Penicillin Binding Protein 1b as a 90kd protein conclusively demonstrates that this is not the case (Nakagawa and Matsuhashi 1982). The *popC* gene is involved in porphyrin biosynthesis, specifically δ-aminolevulinate (Powell *et al.* 1973), but other than this it has not proved possible to obtain further information concerning either the gene, its exact location, or its protein product. Again, the metabolic function of the gene product would tend to argue against it be involved in the regulation of plasmid copy number. Furthermore, its location on the *E.coli* genetic map can only be taken as approximate due to the antiquity of its mapping with regard to the later mapping of adjacent markers. The only remaining genetic marker located between *panB* and *dapD* is *optA*, and this is the only genetic marker whose phenotype would suggest it may be allelic to *pcnB*. The mnemonic *optA* stands for one point two, and it refers to the fact that mutants in the gene 1.2 of coliphage T7 are unable to grow on an *E.coli* host that is mutant in this gene also (Saito and Richardson 1981). Thus a mutation in both phage and host is necessary for this phenotype to be expressed. The interesting aspect about the phenotype of such a double mutant is that the replication of the phage DNA is impaired, while host DNA synthesis is unaffected. In fact this is the only detectable phenotype of the *optA* mutation. In common with the observations made for *pcnB*, no detectable difference in host cell metabolism is seen. The fact that the phage gene 1.2 must be mutant for this phenotype to be expressed would suggest that it encodes its own protein similar to the host *optA* gene product, but can utilise the host protein if required. However, *optA* has been reported as being 92% cotransducible with *dapD* (Saito and Richardson 1981), a considerably higher frequency than that observed for *pcnB*. Additionally, a T7 phage deleted for gene 1.2 was tested to see if growth was affected in a *pcnB* strain, as would be expected if *pcnB* were allelic to *optA*. This showed no reduction in viability compared to controls, indicating that *pcnB* is unlikely to be allelic to *optA* (data not shown).

The above data would tend to suggest that *pcnB* is in fact a newly identified gene rather than simply allelic to a previously discovered marker. Furthermore, utilisation of restriction map data has enabled precise localization of *pcnB* with regard to the neighbouring markers *panB, fhuA, fhuC, fhuD, fhuB* and *dapD* (figure 7.13). It remains to be seen where ~~other~~ the other markers are located on the *E.coli* physical map; for example there is no definative evidence that they are located between *panB* and *dapD*.

Having discussed the possibility that *pcnB* may be allelic to a previously identified *E.coli* gene, the replication control systems of the plasmids tested in this study will now be assessed, in order to assign a possible function to the *pcnB* gene product with regard to the control of plasmid copy number.

7.13.2 Replication or replication control?

In considering how *pcnB* might affect the copy number of various plasmid replicons it is important to differentiate the replication of the plasmid DNA from the actual mechanisms controlling such replication. For example, a reduced plasmid copy number might be due either to a reduced rate of plasmid replication (such as might be seen if a DNA polymerase were mutated, although none of the unmapped subunits of DNA polymerase III are 48kd in size; Maki and Kornberg 1988), or alternatively, as a result of changes to the controlling system such that a reduced frequency of initiation is occurring. In practice however, the latter system is the most plausible route to a low-copy phenotype. Since it takes but a fraction of the cell cycle to replicate even a large plasmid such as R1, a reduced rate of replication could not realistically result in the observed low-copy phenotype of the *pcnB* mutant (for review see Nordstrom *et al.* 1984). For example, replication of the ColE1-type plasmid ClodF13 takes less than 90 seconds under normal growth conditions, and this replication time is unchanged in copy number mutants, indicating that copy number is principally dependent upon the frequency of initiation rather than the time taken to replicate (Veltekamp and Nijkamp 1976). Additionally, chromosomal mutations which significantly reduce plasmid DNA synthesis might be expected to exert a commensurate effect upon the host. Since all the plasmids reviewed here use the host enzyme DNA polymerase III for the synthesis of their DNA, given the crucial role of this enzyme in host cell DNA metabolism (Gefter *et al.* 1971), it would appear unlikely that the *pcnB* mutation would affect expression or activity of this enzyme. Additionally the range of host enzymes used for the DNA synthesis of all these plasmids, whether PcnB-requiring or not, is fundamentally the same (for review see Scott 1984).

7.13.3 Control of ColE1-type plasmid replication

The control of ColE1-type plasmid replication is probably the most intensively studied of all plasmid replicons, due in part, no doubt, to the widespread use of the ColE1 replicon as the basis of many cloning vectors (see Pouwels *et al.* 1985). ColE1, together with the closely related plasmids p15A (Cozzarelli *et al.* 1968), and pMB1 (the progenitor of the pBR-series of plasmids; Bolivar *et al.* 1977a, Lacatena and Cesareni 1983), are characterised by their ability to replicate in the absence of *de novo* protein

synthesis (for review see Veltkamp and Stuitje 1981). This property, together with their small size, has contributed to their popularity as cloning vectors. The addition of protein-synthesis inhibitors such as chloramphenicol results in a cessation of growth and chromosome replication, although plasmid replication can continue. As a result the plasmid copy number is 'amplified' with respect to the bacterial chromosome, often by as much as 50 fold (Clewell 1972).

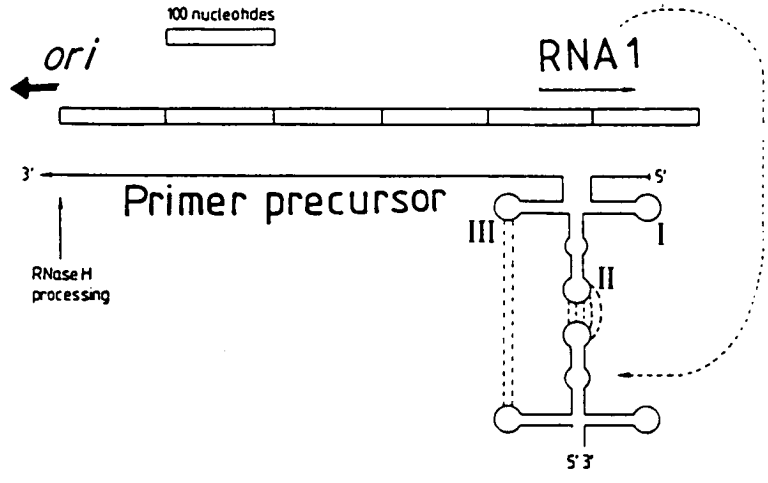
ColE1 is entirely dependent upon *E.coli* specified proteins for its replication (Tomizawa *et al.* 1974). However, with the possible exception of the *pcnB* gene product, *control* of replication appears to be exclusively the preserve of plasmid specified factors. The replication control systems of ColE1 are fundamentally highly similar to those of plasmid R1 (section 7.13.5), comprising both positively and negatively acting RNA elements together with a secondary system utilising a negatively acting repressor protein. Of interest is the fact that a similar control system is used to maintain two quite different plasmid types; R1 being a large, low-copy, conjugative plasmid, while ColE1 is a small, high-copy, non-conjugative plasmid (reviewed in Scott 1984).

Replication of ColE1 is unidirectional from a specific origin, both *in vivo* (Inselburg 1974) and *in vitro* (Tomizawa *et al.* 1974). The first stage in the initiation of ColE1 DNA replication is the synthesis of a primer RNA transcript (RNA II), begun 555bp upstream of the origin of replication which forms a hybrid with the template DNA near the replication origin (Itoh and Tomizawa 1980). Since this event is rifampicin sensitive it seems likely that *E.coli* DNA-dependent RNA polymerase synthesises this primer transcript (Sakakibara and Tomizawa 1974). *In vitro* work has shown that these RNA:DNA hybrids serve as a substrate for RNAaseH, which cleaves the RNA moiety of the RNA:DNA hybrid to produce the RNA primer to which deoxynucleotides are added directly by the host enzyme DNA polymerase I (Itoh and Tomizawa 1980). After a short leading strand is synthesised, subsequent DNA synthesis proceeds by a different mechanism, independent of DNA polymerase I and utilising the host enzyme DNA polymerase III for the bulk of the plasmid DNA synthesis (Tomizawa 1975).

The initiation of ColE1 DNA replication is thus dependent upon the formation of the stable RNA:DNA hybrid, and this event is under the negative control of a small anti-sense RNA species called RNA I (Hashimoto-Gotoh and Inselburg 1979, Tomizawa *et al.* 1981). Synthesis of the ColE1 RNA I starts 445bp upstream of the replication origin and proceeds in the opposite direction to that of RNA II, terminating near the

initiation site of RNA II synthesis (Itoh and Tomizawa 1980). Thus RNA I is anti-sense, and complementary to, RNA II. Both RNA species are capable of forming stem-loop secondary structures which facilitate formation of a stable pairing between them (Lacatena and Cesareni 1983, Tomizawa 1985). This pairing results in conformational changes to the primer transcript (RNA II) which inhibit it from forming a stable RNA:DNA hybrid, and primer formation is prevented (Masukata and Tomizawa 1986). This is shown diagramatically in figure 7.14.

FIGURE 7.14 Replication control of ColE1



Replication region of ColE1. The direction of DNA replication is indicated by the dark arrow, which starts from 'ori'. The open bar represents the DNA, and the vertical bars indicate distances of 100 nucleotides. The transcripts are indicated as arrows above the DNA. The cloverleaf structures for RNA I and the primer precursor are indicated; the postulated hydrogen bond interactions between them are shown as dotted lines. Taken from Scott (1984).

From this, it can be seen that factors which affect either the binding of RNA I to RNA II, or their rates of synthesis might be expected to exert an effect upon the copy number of ColE1. Indeed, mutations which remove or alter parts of the RNA I sequence result in an altered plasmid copy number, as would be expected from changes in the efficiency of RNA I: RNA II binding (Moser and Campbell 1983, Meusing *et al.* 1981). It is this interaction which is the basis for the incompatibility in the ColE1 group of plasmids (Tomizawa and Itoh 1981, Som and Tomizawa 1983). ColE1-type plasmids of a different incompatibility group have a different nucleotide sequence for the RNA I-RNA II region, although the molecules can be folded into analagous secondary structures (Som and Tomizawa 1982).

ColE1 specifies another product involved in the regulation of copy number in addition to RNA I. This is the *rop/rom* gene product, a 63 amino acid peptide which acts to negatively regulate plasmid copy number (Twigg and Sherratt 1980, Tomizawa and Som 1984). In this it is roughly analogous to the CopB protein of plasmid R1, which represses transcription of the initiator RNA (section 7.13.5). The mode of action of the Rop/Rom protein is somewhat different however. Rather than repressing the expression of the positively acting initiator element as per CopB, it serves to enhance the binding of the anti-sense RNA I to the RNA II primer transcript (Tomizawa 1986). The result is a negative effect on plasmid replication, and ColE1 derivatives deleted for this region demonstrate a commensurate increase in copy number (Twigg and Sherratt 1980). However the copy number effect seen in a *pcnB* strain must be independent of the Rop/Rom protein effect, since the reduction occurs with both rop^+ and rop^- plasmids (plasmid pBR328, the progenitor of pJM511, pJM513 and pJM516 is rop^-; pBR325 is rop^+. Both parent plasmids are affected in a *pcnB* host).

However, in addition to the above controls on plasmid replication, it has recently become clear that in the absence of certain host-encoded proteins, several alternative initiation systems seem to function. In spite of the seemingly crucial role played by DNA polymerase I and RNAaseH in the initiation of ColE1 replication, it has been discovered that plasmid ColE1 can be stably maintained in bacteria lacking RNAaseH (Naito *et al.* 1984). Additionally, it has become apparent that in a host lacking RNAaseH, DNA polymerase I, which was previously found to be essential for the maintenance of ColE1 (Kingsbury and Helinski 1970, 1973b) has now become dispensable (Kogoma 1984). Although successful replication of ColE1 in the absence of RNAaseH and DNA polymerase I suggests the use of an alternative replication system, this replication still requires the presence of the intact ColE1 replication origin (Kogoma 1984), and perhaps more significantly, for synthesis of RNA II to occur (Naito and Uchida 1986). Work by Dasgupta *et al.* (1987) has in fact suggested the presence of at least two alternative mechanisms for the initiation of ColE1 DNA replication in the absence of DNA polymerase I and RNaseH, although both mechanisms still require the presence of the RNA II:DNA hybrid at the replication origin. In addition, the inhibitory effect of the RNA I binding to RNA II is still present in such a system, suggesting that the actual controlling mechanisms have remained unchanged. Basically it appears that in the absence of RNAaseH, RNA II remains hybridised beyond the origin region, displacing the non-transcribed strand and making it available for DNA synthesis (i.e. the opposite strand to normal is used for priming). In the absence of DNA polymerase I (and RNAaseH) it appears that the uncleaved RNA II transcripts are able to act as primers for DNA synthesis in the normal direction. For the sake of

brevity, these alternative systems shall not be discussed here, and the reader is directed towards the work of Dasgupta *et al.* (1987) and Masukata *et al.* 1987 for further information. The last piece of evidence concerning the heterogeneity of ColE1 DNA replication mechanisms concerns the work of Seufert and Messer (1987), who found that in the absence of protein i, the addition of DnaA protein was able to substitute for primosomal assembly in pBR322 DNA replication *in vitro*. However, it is not clear whether this substitution is actually affecting the controlling mechanisms or simply the physical processes involved in replication. Clearly though, it would seem that ColE1-type plasmids are able to demonstrate considerable versatility in the use of host enzymes to fulfil their DNA replication requirements, and it would thus be of some interest to discover if the events occurring at a molecular level in a *pcnB* strain are in any way altered.

7.13.4 Control of ColD replication

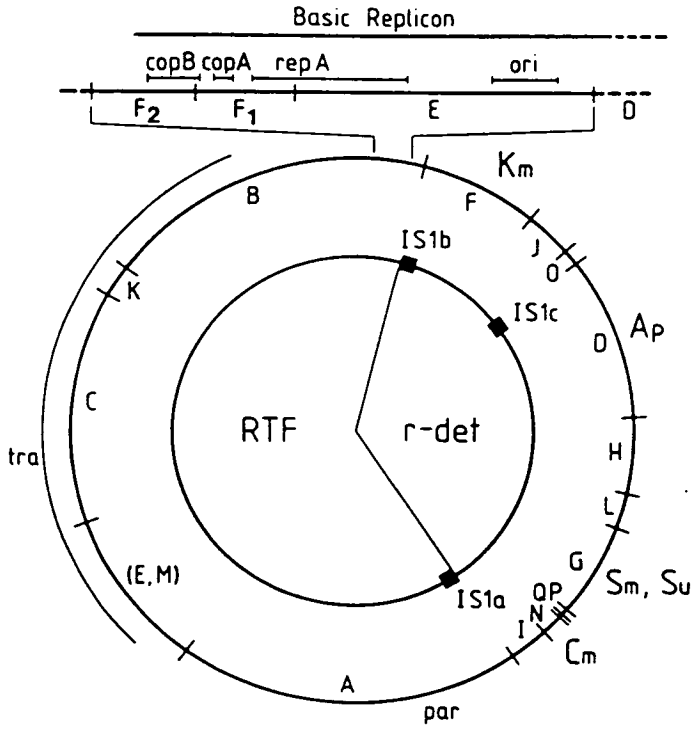
Several cloning vectors based upon the ColD replicon exist, and their main usefulness lies in the fact that they are of a different incompatibility group to the vast majority of the pMB1-based cloning vehicles (Pouwels *et al.* 1985, Frey and Timmis 1985). Such vectors, included the plasmid pVH1 as tested in this study, are derivatives of the naturally occurring *E.coli* plasmid ColD-CA23, which codes for Colicin D, a peptide which acts to inhibit protein biosynthesis in sensitive cells of *E.coli* (Timmis and Hedges 1972). Unfortunately a paucity of information exists on the ColD plasmid, with essentially no detailed information concerning its replication control system. What information there is would tend to suggest that ColD is probably related to the ColE1 group of replicons, although this can by no means be taken for granted. Like plasmid ColE1, ColD encodes a bacteriocidal colicin, and its small size (4.63kb; Timmis *et al.* 1978) and relatively high copy number (20 copies per cell: Frey and Timmis 1985) are similar in nature to ColE1. Additionally, ColD appears to undergo an increase in copy number as the host strain goes into stationary phase in common with the effect noted with ColE1, although this increase appears to be more marked with ColD (Frey and Timmis 1985, Stueber and Bujard 1982). The only information regarding ColD DNA replication is a requirement for the host-encoded enzyme DNA polymerase I (Frey and Timmis 1985), again similar to the requirement seen with ColE1. Apart from this limited information nothing else appears to be known at the present time. Although what information there is would tend to suggest that ColD is probably a ColE1-type plasmid, it leaves no room for speculation as to the mechanism of the dependence of ColD on *pcn*. In the absence of further information it seems plausible that this dependence originates in the same way as that of ColE1.

7.13.5 Control of R1 Replication

R1 is a large conjugative plasmid of approximately 90kb in length encoding multiple antibiotic resistances (for review see Nordstrom *et al.* 1984). Despite its large size however, all of the genes required for controlled replication, including the origin of replication, are clustered in a 2.5kb region located near Insertion Sequence 1b (see figure 7.16). Mini-R1 derivatives, such as pGW71 (Larsen *et al.* 1984) which carry only this region, are able to replicate autonomously with a copy number similar to R1 itself (4-5 copies per cell; Kolleck *et al.* 1978). Both of the R1-derivatives tested in this study, pKN500 (Molin *et al* 1979) and pGW71 (Larsen *et al.* 1984) carry this minimal origin; however, in the case of pKN500 this is augmented by several kilobases of DNA to either side. This DNA includes some of the genes of the *tra* operon (involved in conjugative transfer; Achtmann *et al.* 1978), together with the kanamycin resistance gene from the r-determinant region (Kopecko *et al.* 1976). The region of R1 DNA contained within each plasmid is thus shown in figure 7.15. The requirement for *pcn* does not appear to be connected with the *par* region of R1, involved in stable partitioning of the plasmid, since this region is lacking in both R1-derivatives.

Regulation of R1 replication concerns the products of the *copA, copB* and *repA* genes, all of these being present on the minimal origin as described above (see figure 7.15). The product of the *repA* gene seems to be the key element in determining the frequency of initiation from the R1 origin, acting as a rate-limiting positive factor in the initiation event (Light and Molin 1981). The products of the *copA* and *copB* genes act to negatively regulate expression of *repA*, and thus maintain an overall control over plasmid copy number. The product of the *copA* gene is a small RNA molecule which acts as an anti-sense RNA by binding to and inhibiting translation of the *repA* mRNA (Light and Molin 1983). CopA is produced constitutively, and binds to a region on the *repA* mRNA upstream of the actual coding sequence, this region being designated *copT* (Danabara *et al.* 1981). Binding of CopA to CopT results in the formation of a duplex molecule which leads to the inhibition of *repA* mRNA translation, although the mechanism of this inhibition is at present unclear (Gerhardt *et al.* 1987). This is similar to regulation of ColE1 replication which also utilises a small anti-sense RNA; however, in this instance the initiator molecule is the RNA itself rather than its protein product. A further similarity to ColE1 regulation is provided by the CopB protein of R1, which in common with the Rop/Rom protein of ColE1 acts as a negative regulator of copy number, additional to the action of the antisense RNA. The product of the *copB* gene is a constitutively expressed 10.5kd protein which binds to the *repA*

FIGURE 7.15 Replication control of R1



Genetic and physical map of plasmid R1. RTF and r-det refer to resistance transfer factor and resistance determinant respectively. IS1a, IS1b, and IS1c refer to insertion sequences. Capital letters A-Q refer to fragments generated by restriction endonuclease EcoRI. Plasmid pKN500 contains fragments B and F, while plasmid pGW71 contains the minimal origin only. Phenotypic symbols Ap, Cm, Km, Sm, and Su refer to resistance to ampicillin, chloramphenicol, Kanamycin, streptomycin and sulfonamide respectively. Genetic symbols are cop (copy number control), ori (origin of replication), par (partitioning), rep (replication), and tra (transfer).

promoter and thus represses transcription of the *repA* gene (Light and Molin 1982, Riise and Molin 1986). Regulation is thus exerted at both a transcriptional and a translational level. Control of R1 copy number can therefore be seen as depending upon the ratios of the *copA, copB* and *repA* gene products, and factors which affect this ratio should consequently exert an effect upon the plasmid copy number. Theoretically then, mutations which both increase and decrease R1 copy number should exist, and in practice both types have indeed been reported. Chromosomal mutations which either reduce (Kingsbury and Helinski 1973a; Kingsbury *et al.* 1973) or increase (Cress and Cline 1976) R1 plasmid copy number have been reported; neither class appears to have been given a chromosomal location, nor has their mode of action been elucidated. Plasmid located mutations so far isolated appear only to result in an increased R1 copy number, although this is probably due more to the method of selection rather than any predisposition towards occurrence of this type. For plasmid

located high-copy mutations, increases due either to increased transcription of the *repA* gene (caused by insertion of a strong foreign promoter fragment; Larsen *et al.* 1984), or mutations which reduce the expression of the negatively acting *copA* and *copB* gene products (Uhlin *et al.* 1979) have been reported. Mutations which decrease *repA* expression or increase that of *copA* or *copB* might be expected to reduce the copy number of R1; to date neither type have been reported. *E.coli* proteins which are required for R1 replication include the products of the *dnaB, dnaC, dnaE* (DNA polymerase III subunit) and *dnaG* genes (Rownd 1978). It is unclear whether R1 requires the *dnaA* gene product (Molin and Nordstrom 1980; von Meyenburg and Kogoma, quoted in Hansen and Yarmolinsky 1986, Nagata *et al.* 1988). Although plasmid ColE1 requires DNA polymerase I (the product of the *polA* gene), this is not required for R1 replication (Kolleck *et al.* 1978).

7.13.6 F, pSC101 and *oriC* replication control

The bacterial plasmid pSC101 is a naturally occurring small plasmid of 9.4kb in length, encoding tetracycline resistance and originally isolated from *Salmonella panama* (Cohen and Chang 1977). pSC101 has a copy number of about 7 copies per cell (Meacock and Cohen 1980), and thus is intermediate in copy number between high copy number plasmids such as ColE1 and low copy number plasmids such as F. The F plasmid is probably best known as the prototype sex-factor, and it is a large unit copy plasmid of approximately 94.5kb in length (Frame and Bishop 1971). In common with the bacterial chromosome, the low copy number of F would suggest the existance of very precise mechanisms to regulate the control of initiation and segregation and thus prevent plasmid loss from a culture during growth and division.

Several features of the initiation control of plasmids pSC101, F, and at *oriC* are highly similar, and therefore the three replicons shall be considered together. While replication control of R1 and ColE1-type plasmids appears to reside with separate positively and negatively acting elements (the latter serving to limit expression of the former), replication control of F, pSC101 and at *oriC* appears to reside in a single positively acting 'initiator' protein, which additionally serves to repress its own transcription (and is thus autoregulatory). Such a role for an initiatior protein would suppose binding sites both at the replication origin (where it acts as a positive factor) and at the promoter of the gene itself (where it would serve to repress its own expression and thus act as a negative element). Additionally, preferential binding at the site repressing its own synthesis would be required if runaway replication were not to occur.

Replication from *oriC* is absolutely dependent upon the *dnaA* gene product (Hirota *et al.* 1968, Fuller *et al.* 1981), and several lines of evidence suggest that this protein fulfils the role of a positively acting initiator element, since increased expression of *dnaA* results in an increased frequency of initiation from *oriC* (Atlung *et al.* 1987, Xu and Bremer 1988). Additionally, DnaA binds both at *oriC* (Fuller *et al.* 1984) and is autoregulatory (Atlung *et al.* 1985a, Braun *et al.* 1985). Similarly, replication of both F (Hansen and Yarmolinsky 1986) and pSC101 (Hasunuma and Sekiguchi 1977, Frey *et al.* 1979) is dependent upon the *dnaA* gene product, and in the case of pSC101 at least it appears to act as a positively acting initiator element, since *dnaA* mutants which result in increased chromosomal initiation also have the same effect on the pSC101 initiation frequency (Frey *et al.* 1984). However, it is to be expected that plasmid encoded elements would also play a role in replication control, otherwise runaway plasmid replication would take place. In fact both pSC101 and F encode their own positively acting initiator protein.

For plasmid pSC101, this protein is the product of the *repA* gene, which appears to act both as a positive factor in initiation control, while at the same time repressing its own transcription (Linder *et al.* 1985, Armstrong *et al.* 1984). Additionally, lower levels of *repA* are required for autoregulation than are needed for initiation (Linder *et al.* 1985). Binding of RepA at the pSC101 origin appears to be co-operative in nature (Vocke and Bastia 1983), in common with the binding of DnaA at *oriC* (Kaguni and Kornberg 1984). For F, this role is fulfilled by the *E* protein, which again is both positively acting in initiation control, and negatively acting in control of its own expression (Sogaard-Andersen *et al.* 1984, Rokeach *et al.* 1985). The structure of the origins is remarkably similar in each instance also. Both the pSC101 and F origins contain AT-rich regions followed by direct repeats of 18 and 19 base pairs respectively, the binding sites of the RepA and *E* proteins (Churchward *et al.* 1983, Murotsu *et al.* 1981). Similarly, *oriC* contains a series of 9 base pair repeats, the binding sites of the DnaA protein (Meijer *et al.* 1979, Fuller and Kornberg 1983). A further similarity is the presence of DnaA binding sites in both the F and pSC101 origins, and in the latter instance at least, DnaA has been shown to bind to this sequence *in vitro* (Fuller and Kornberg 1983)

However, the question as to why pSC101 and F should depend on DnaA in addition to their own positively acting initiator element deserves consideration. It is possible that both 'initiation elements' are required for initiation to take place, although changes in only one may increase or decrease the frequency of this occurring. For example, the *repA* or *E* proteins may be involved in controlling the actual frequency of

initiation, with DnaA itself being involved in the physical processes of initiation.

Potential copy number mutants of F or pSC101 might be expected to result from changes in the expression of *dnaA* or the *repA* and *E* genes. Chromosomal mutations which result in both reduced and increased copy numbers of F have been reported; however, neither the chromosomal locations of these mutations, nor their physiological basis have been investigated (Kingsbury and Helinski 1973a, Kingsbury *et al.* 1973, Cress and Cline 1976). As far as I can ascertain, no chromosomal mutations which affect pSC101 copy number (with the exception of *dnaA*) have been reported.

In the light of the similarities between F, *oriC*, and pSC101 replication control, it would appear suprising that only the latter appears to be affected by the *pcnB* mutation. Given the similarities in replication control between F and pSC101, how might pSC101 resemble the otherwise dissimilar ColE1 in being *pcnB* dependent? At least two possible explanations appear tenable. F may encode its own *pcnB* analogue, thus rendering the chromosomal *pcnB* unnecessary. Alternatively, F replication may be independent of PcnB while that of pSC101 requires it. However, the similarity between F and pSC101 replication control would tend to argue against the latter hypothesis, while the large size of F in comparison to pSC101 (100kb against 9kb) would certainly leave room for additional undiscovered replication genes. This possibility could be examined by testing mini-F derivatives to see if removal of DNA had resulted in them becoming *pcnB*-requiring. An alternative possiblity might be that pSC101 does encode a PcnB-type protein, but that the derivative examined in this work (pPM30) has lost this function in construction and is now dependent upon the chromosomally encoded PcnB. Since the pPM30 only contains 2.9kb of pSC101 DNA, this hypothesis is certainly plausible, and would perhaps suggest testing of the full sized pSC101 for a requirement for *pcnB*.

7.13.7 Summary and conclusions

The replicons examined in this study (p15A, pMB1, ColD, R1, F and pSC101) appear to fall into two distinct catagories as regards replication control. In the first group, which includes the ColE1-type plasmids pMB1 and p15A, together with the large conjugative plasmid R1, three separate control elements seem to function. These include a positively acting 'initiator' transcript, a negatively acting anti-sense RNA which binds to this transcript, and a third component comprising a negatively acting protein which interacts at the plasmid origin. However, although superficially highly similar, ColE1 and R1 replication control differ in several details. In ColE1 replication the positively acting initiator is the RNA transcript itself; in R1 replication it is the

proteins located at the vector-insert junctions. An autorad showing the resulting radiolabelled protein products is shown in figure 7.10a. Besides the β-lactamase proteins, 4 strong protein bands unique to pJM513 are present in the undigested template track. The estimated molecular weights of these proteins are 48kd, 28kd, 17kd and 9kd. The 9kd protein is absent from the digested pJM513 template track, indicating that this is a fusion protein and not encoded by the insert fragment (the strength of the band suggesting that it is transcribed from the chloramphenicol promoter. This is fact proved to be the case, as this protein is still present if the *Hin*dIII end of the insert fragment is removed; data not shown). Therefore the proteins of 48kd, 28kd and 17kd molecular weight appeared to be possible candidates for the *pcnB* gene product.

Further characterisation was possible following construction of pJM511 and pJM516. *In vitro* translation and subsequent SDS-polyacrylamide gel electrophoresis using these DNAs as templates yielded the autorad shown in figure 7.10b. These show the 28kd and 17kd proteins to be encoded by pJM511, with the 48kd protein being encoded by pJM516.

Is this 48kd protein the *pcn* gene product? Later work conducted in this laboratory has almost certainly demonstrated that this is so. Examination of figure 7.7 shows the presence of 2 *Kpn*I sites separated by 400bp of DNA in pJM516. Deletion of this region from pJM516 (Damon Hart-Davies) results in a plasmid which is *pcn*$^-$ (data not shown). Work by Micheal Colloms in this laboratory has indicated that deletion of part of this DNA (from the *Kpn*I site at 5690bp in pJM516 to the *Hin*dIII site) results in a truncation of the 48kd protein of pJM516. Therefore this protein is almost certainly the *pcn* gene product. Furthermore, this shows the direction of transcription of *pcnB* to be from the *Eco*RI site towards the *Hin*dIII end of the insert (figure 7.7).

7.9 A comparison of *pcn* with other low copy mutations

As has been mentioned in the introduction to this chapter, the designation *pcn* to describe the low copy mutation originally isolated in JM21 was adopted following the publication during the course of this work of a paper describing a similar low copy mutation by Lopilato *et al.* (1986). This mutation had also been mapped to approximately 3 minutes on the *E.coli* genetic map, and the authors suggested the mnemonic *pcnB* (plasmid copy number) as a suitable gene name. The similarity of map position suggested that both mutations might map to the same gene or operon. The mutant described by Lopilato *et al.* had been isolated using essentially the same

principle as yielded JM21; that is, the reversal of a deleterious effect consequent upon the cloning of a gene in high copy. A strain carrying *pcnB* (Mri93) was obtained and tested to see if the low copy mutation was allelic to that described in this work. Another low copy mutant, WT286 was also tested. This strain had been obtained from W. Tacon and was known to maintain pBR-type replicons at a reduced copy number, but apart from that nothing was known about its method of isolation or the map position of its mutation.

Both Mri93 and WT286 were tested using the λ clones originally used to map the *pcn* mutation of JM21/JM18. Mri93 was transformed with pBR325 and p*cI*^{857} and tested for complementation as described in section 7.7. WT286 was transformed with the pBR325 derivative pPE13 and p*cI*^{857} followed by complementation testing as per Mri93. Plasmid pPE13 carries the *recA* gene of *E.coli*; since WT286 is *recA* and the phage are *att*$^{-}$, this is required for integration of these phage via homologous recombination. The results of these complementation tests showed that the low copy mutations of both Mri93 and WT286, in common with that of JM21/JM18 could be complemented by λ clones number 115 and 116. This suggested that all three mutations were either allelic or else very closely linked. The construction of pJM516 allowed a further confirmation of this. Transformation of Mri93, WT286 and another Tacon low copy strain, similarly uncharacterised and called WT264, showed all four to be complemented by this plasmid and thus probably allelic (Michael Colloms, pers. comm.). As such, I shall hereafter refer to each allele by the strain number in which it was tested or isolated (WT286 *pcnB286*; WT264 *pcnB264*; Mri93 *pcnB93*; JM18/JM21/MM28-2 *pcnB18*).

Although all *pcn* mutations appear to be allelic, the extent to which the copy number is reduced appears to vary depending upon the allele (host strain differences may play a part also since these strains are not isogenic). This is most easily seen when comparing the ampicillin resistance of pBR325 in Mri93, WT286 and JM18. Although only JM18 and MM18 are isogenic and thus strictly speaking comparable, this does give a general indication of the effect of each *pcnB* allele. A mid-log phase culture of each strain was diluted down to give c. 10^3 colonies per plate, these being plated onto a variety of different concentrations of ampicillin and the number of colonies present on each plate after incubation counted. The results are shown graphically in figure 7.11, with the number of colonies present on the control (no ampicillin) plate being taken as representing 100% viability (approximately 600 colonies). The high level of ampicillin resistance seen with MM18 pBR325 (linear up to

protein product of this RNA transcript. In ColE1 replication the anti-sense RNA acts to prevent the initiator RNA from being used as a primer; in plasmid R1 it acts to prevent translation of the RNA. Finally, with ColE1 the negatively acting protein increases the efficiency of binding of the anti-sense RNA to the primer transcript; for R1 it represses transcription of this primer mRNA.

The second group, which includes F, pSC101 and *oriC* appear to utilise a single positively acting initiator protein in their replication control. To prevent runaway replication, this protein is autoregulatory and represses its own expression, such that changes in copy number are self-correcting by virtue of the commensurate change in the protein concentration. Members of this class are both Pcn-dependent and Pcn-independent. However, since the pSC101 derivative tested in this study contained only the replication origin, it is possible that the full-size plasmid may be independent of this requirement. The data on the various plasmids is summarised in table 7.13.

TABLE 7.13 Summary of the requirement by various plasmid replicons for the *pcnB* gene product

Plasmid/Origin	Class[a]	*pcn* -requiring
oriC	I	NO
ColE1	II	YES
ColD	? (II)	YES
p15A	II	YES
pSC101	I	YES
F	I	NO
R1	II	YES[b]

a. Type of replication control. Class I origins utilise a single positively acting initiator protein which also acts to regulate its own expression. Class II replicons utilise both positively and negatively acting RNA species. See text for full details.

b. Unclear whether larger R1 plasmid requires the pcnB gene product.

Since *pcnB* mutants result in a reduced plasmid copy number this would tend to suggest that PcnB is somehow involved in events which determine the frequency of initiation. However, there appears to be no apparent correlation between a

requirement for PcnB and any particular system of replication control. Could *E.coli* encode its own system to regulate plasmid copy number, additional to that of the plasmid? For example to maintain plasmid copy number at an acceptably low level so as not to place an undue burden upon the cells metabolism? Could *pcnB* be mutant in such a regulatory system? However, the fact that *pcnB* is a recessive allele would tend to argue against such a hypothesis, and instead suggest that some limiting factor is causing the observed reduction in copy number. For example, with plasmid ColE1 this might be an RNAase, such that primer formation would be reduced. However, this could not explain the effect of *pcnB* upon other replicons, since this feature of replication control is absent from the others. Additionally, the fact that two previously compatible ColE1-type replicons (p15A and pMB1) have now become incompatible would further suggest that some limiting factor is resulting in the observed phenotype. Are there any features common to the replication control of all replicons which would allow correlation with a Pcn-requirement?

On a gross level, it would appear that the plasmids which are Pcn-independent, F and possibly R1, are large conjugative types, while those requiring PcnB are generally small. Unfortunately this appears to be the only feature to correlate with a requirement for PcnB. The similarities in replication control between the various plasmids would suggest that the former types possibly encode their own PcnB-type protein, removing the requirement for the the chromosomally encoded PcnB, rather than their replication origins being Pcn-independent. This may explain the situation seen with R1; the mini-R1 derivative (pGW71) being dependent upon PcnB, while the larger pKN500 possibly being independent. This would imply the loss of a plasmid-encoded PcnB analogue in the mini-R1 derivative. This is somewhat similar to the situation seen with the *E.coli optA* mutation discussed in section 7.13.1, where phage T7 only requires the *E.coli optA* gene product if it carries a mutation in its own gene 1.2. Thus T7 presumably encodes its own OptA analogue, but can utilise the chromosomally encoded OptA protein if required. Thus both a T7 gene 1.2 mutation and an *E.coli optA* mutation are necessary for the phenotype to be present. This may explain the situation seen with the R1 plasmids.

If this is indeed the case, and explains the pattern of *pcn* dependence seen in table 7.13, the question of what the *pcnB* gene product actually is remains to be answered. Why for example does *E.coli* appear to be independent of PcnB? Is it a plasmid derived gene which has become transposed into the *E.coli* genome and is exclusively for the use of resident plasmids? This is probably not the case, since probing to the DNA of a variety of enteric bacterial species has revealed a level of

homology for *pcnB* similar to that seen for nutritional markers, and certainly much higher than that seen for recently acquired phage markers (Ian Oliver, pers comm.). If *pcnB* is thus a reasonably well conserved, possibly essential *E.coli* gene, what might its function be?

For want of any evidence to the contrary I would like to suggest that *pcnB* may encode an *E.coli* RNA polymerase sigma factor, or possibly a primase analogue, specifically used for plasmid replication. Since replication of all plasmids requires some form of transcription, be it to synthesize the primer RNA to which deoxynucleotides are directly added, as per ColE1, or whether it is the transcription of the positively acting 'initiator' protein, as per R1, pSC101 or F, this is a common feature which may be affected in a *pcnB* strain. With the exception of ColE1 and *oriC* replication, although a fair amount is known about the mechanisms *controlling* replication, little is known about the actual biochemical events during the initiation of replication, and it is probably fair to assume that all plasmids require transcription of a primer RNA to which deoxynucleotides can be added. Assuming a host enzyme specific for this process, it can be seen that mutations in this gene would exert an affect upon plasmid replication whilst leaving host DNA replication unaffected.

7.13.8 Suggestions for further study

At such an early stage in the characterisation of a gene a multitude of possibilities for further study present themselves. In relation to the above mentioned hypothesis that *pcnB* may encode an enzyme involved in RNA synthesis, this could be examined at a later stage in its characterisation by comparing protein sequences etc. and searching for homologies, or examining replication of sensitive plasmids *in vitro* using extracts from a *pcnB* strain.

The first experiments would probably be concerned with examining null mutants of *pcnB*, probably isolated by insertional activation. The successful isolation of such mutants would demonstrate the non-essential nature of the *pcnB* gene. Further obvious experiments would be to sequence the *pcnB* gene. Comparison with the database should allow identification of features common to other proteins, for example DNA binding domains, RNAases etc. Replication of plasmids *in vitro* using extracts from a *pcnB* strain should allow further characterisation of the effect of this mutation. For example, at which stage of the replication process the *pcnB* gene product acts.

To examine the possibility that larger plasmids may encode their own

Pcn-type protein, it should be possible to probe to their DNA using purified *pcnB* DNA and look for homologies. Since mini-R1 requires PcnB in possible contrast to the full size plasmid, it should be possible to examine various deletion derivatives of R1 to narrow down the region in which this Pcn-independence lies. Obviously this could then be examined to see if it encodes a similar protein type etc. This type of examination could be conducted for the F plasmid and other Pcn-independent replicons. Purification of the PcnB protein, possibly by utilising a *b*-galacosidase fusion approach followed by the raising of antibodies should be useful on two counts. Firstly, the antibodies could be used to test the above plasmids for cross-reactivity of plasmid-encoded PcnB-type proteins (if present). Secondly, use of such antibodies to inhibit stages of plasmid replication in an *in vitro* replication system should give some indication as to the stage at which the *pcnB* gene product acts. Additionally, they could be used to determine whether the *pcnB* gene product is normally present in the repertoire of intracellular proteins or whether it is only expressed when the cell is acting as host to a colonising plasmid.

In addition to further characterisation of *pcnB*, investigation of further copy number mutants may prove fruitful. As has been discussed earlier (section 7.12), the use of the *groE* over-expression system to isolate additional copy number mutants is a repeatable phenomenon. Such mutants could be screened to see if other chromosomal loci concerned with plasmid copy number arose. A λ*pcn* lysogen (diploid) should help in this respect. It may be that all such mutants map to the *pcnB* gene. If so, using a deleterious gene-expression system (such as *groE*) cloned into a different plasmid replicon (such as mini-F for example) may well isolate a whole different class of chromosomal genes concerned with copy number control. Additionally, if such an expression system were cloned into an *E.coli oriC* replicon, such copy number mutants may give significant insights into the regulatory elements concerned with *E.coli* replication control. At this point in time perhaps one may be forgiven for supposing that further investigations of the kind begun in this study may prove to be a fruitful line of research indeed.

CHAPTER 8

An Investigation into the Determination of Plasmid Copy Number by the Technique of Quantitative Hybrisation

During the course of the work described in this thesis an accurate measurement of the intracellular plasmid copy number was required on a number of occasions. Although a variety of well-characterised techniques already exist, they appeared to be either too inaccurate or too indirect to be considered suitable for use under the circumstances. As a result of this it was decided to develop the technique of quantitative hybridisation as a means of measuring plasmid copy number. This chapter describes the results obtained, and examines the applications and limitations of the system as a generally applicable experimental technique.

8.1 Introduction: The measurement of plasmid copy number

The measurement of plasmid copy number is important for a variety of experimental purposes. These can include direct investigations into factors involved in the replication of bacterial plasmids, in which effects upon plasmid copy number can be taken as an indication of the involvement of a particular process or gene product. Equally importantly, in a variety of situations where bacterial plasmids are being used as experimental tools it is often necessary to have an indication of the copy number. This is especially true where plasmid-based systems are being used to measure promoter activities under a variety of conditions (e.g. McKenney *et al.* 1981), and changes in plasmid copy number could lead to an incorrect interpretation of promoter strength data. Thus an accurate estimate of the plasmid copy number is often highly desirable.

A variety of techniques exist for the measurement of plasmid copy number, each with its own advantages and disadvantages, such that no single approach can unequivocally be recommended for all applications. However, bearing in mind factors such as the rapidity of assay, together with the required level of accuracy for a particular application, should allow adoption of the most suitable system under most circumstances. For example, when investigating the low-copy mutant described in chapter 7 it was found that antibiotic resistance levels provided a rapid screen for the transductants obtained during mapping experiments. However, quantitative hybridisation was the preferred technique when a more accurate determination of the absolute level of plasmid copy number was required.

Broadly speaking, four general methods of measuring plasmid copy number exist. These are:-

1. Enzymic assay of a plasmid-encoded protein, where synthesis is proportional to gene dosage.

2. Measurement of the level of antibiotic resistance conferred.
3. Curing curves to measure the rate of plasmid loss in the absence of selection (for *par*$^-$ plasmids with low copy numbers).
4. Direct measurement of the level of plasmid DNA

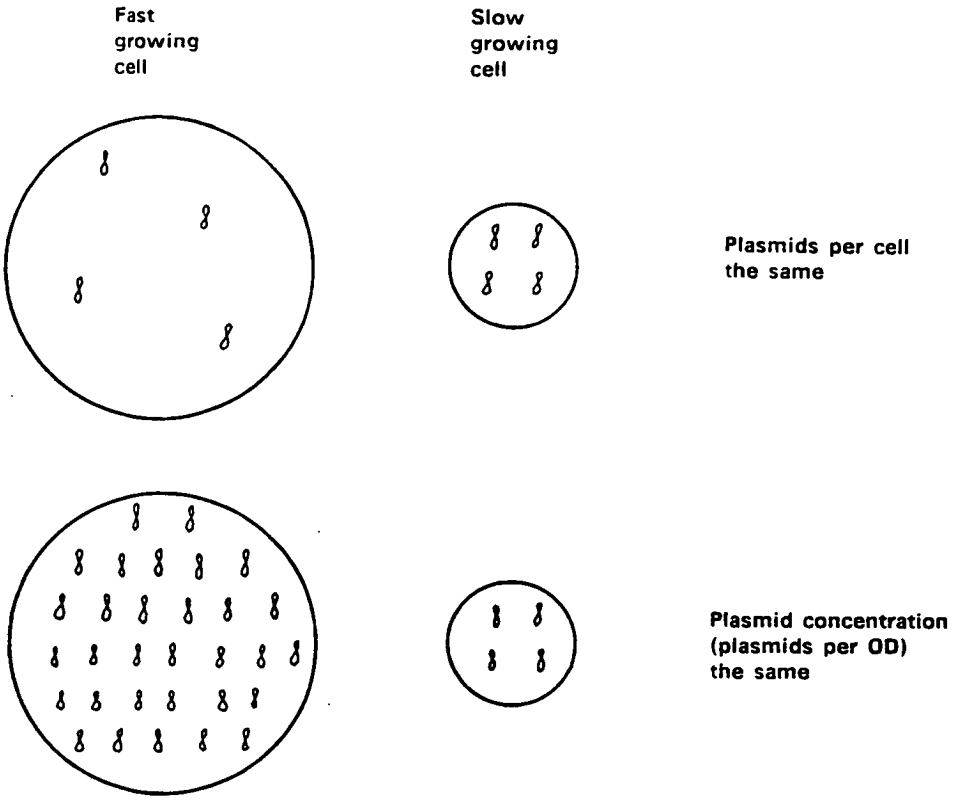
Before a more detailed assessment of each technique is made, the actual meaning of the term 'plasmid copy number' shall be discussed. This is important since the concept of 'plasmid copy number' is rather vague, and tends to mean different things to different people. Obviously for a fuller appreciation of the concept of measuring copy number a more exact definition would be desirable.

8.1.1 A definition of plasmid copy number: plasmids per cell, plasmids per chromosome or plasmid concentration?

Depending on the method of measurement (and to an extent, who is performing the measurement), 'plasmid copy number' can either refer to the number of plasmids per chromosome equivalent, the concentration of plasmids in the culture (i.e. plasmids per O.D. equivalent), or the actual number of plasmid molecules in a single cell. Unfortunately all three definitions tend to be used interchangably, the assumption generally being that they all refer to the same measurement. This is patently not so: indeed, conditions which can result in a change in 'plasmid concentration' over a several-fold range can leave the 'number of plasmids per cell' unaltered. The reasoning behind this is discussed below.

Cell size in *E.coli* varies depending upon the growth rate; at the fastest growth rates (broth cultures), the average size of the individual cell can be up to 8 times as large as that of a cell growing in minimal medium (see Donachie *et al.* 1984 for review). This means that at an $O.D._{540}$ of 0.2, a fixed volume of the cells growing in minimal media will have 8 times as many cells as the same volume from the fast growing culture, although the cellular mass will be the same. Thus in comparing the 'copy number' between different cultures an appreciation of the effect of growth rate on cell size has to be maintained. But how does the copy number vary depending upon growth rate? Does a cell which is 8 times as large contain 8 times as many plasmids as a cell of unit volume? (i.e. The number of plasmids per cell increases eight-fold, although the plasmids per O.D. - plasmid concentration - remains the same). Or, does a cell 8 times the size contain the same number of plasmids as a cell of unit volume (i.e. the number of plasmids per cell remains constant, but the plasmids per O.D. is reduced eight-fold). This is most easily appreciated with the aid of the diagram (figure 8.1). Alternatively, somewhere in between may be the true situation.

FIGURE 8.1 Changes in cell size and the effect on plasmid copy number



The first possibility would seem the most likely if a system such as the repressor dilution model of Pritchard *et al.* (1969) were controlling plasmid replication. This would result in the maintenance of a constant concentration of plasmid molecules; as the cell grew and the constitutively expressed repressor were diluted out, so plasmid replication could occur. However, for ColE1-type plasmids at least this does not appear to be the case. Experimental evidence (J. March unpublished results, Stephen Black unpublished results, Lin-Chao and Bremer 1986) suggests that in fact the number of plasmids in a cell appears to stay relatively constant, irrespective of the cell size. As a result, the plasmid concentration goes down as the growth rate (and hence the cell size) increases. This is an unusual result, and suggests a novel form of control that is independent of concentration effects. The F plasmid appears to be subject to a similar kind of growth rate dependent effect also (Shields *et al.* 1986). It is outside of the scope of this work to consider this in detail here, although such a system of control has been observed for the cell division gene *ftsZ* of *E.coli* (see Donachie *et al.* 1984), leading on to the intriguing possibility that this may represent some form of a

link between DNA replication control and subsequent cellular division.

It is obvious then from theoretical considerations that 'plasmids per cell' and 'plasmid concentration' must change in different manners according to changes in the growth rate. Such changes should therefore be borne in mind when comparing the plasmid copy number between cultures growing at different rates, or indeed in situations where there may be differences in cell size.

The situation regarding 'plasmids per chromosome equivalent' is even more complicated since an extra variable is included in the form of the behaviour of the bacterial chromosome. Thus the question of how chromosomal DNA changes with growth rate must be addressed also. This is perhaps ironic, since plasmids per chromosome equivalent is a commonly used way of expressing plasmid copy number. Essentially it is the ratio of plasmid DNA to chromosomal DNA in a fixed volume of culture, both species being visualised by ethidium bromide staining and/or radiolabelling. Since the molecular weight of the plasmid is known, and that of the chromosome is estimated at 4700kb, the respective molar ratios of each can be easily calculated. However, changes in the concentration of chromosomal DNA with growth rate have to be considered. Unless plasmid and chromosomal DNAs vary in exactly the same manner, any comparison of the plasmid/chromosomal ratio is difficult unless the growth rates of the cultures to be compared are identical. As discussed above, the number of ColE1-type plasmids per cell appears to remain constant, regardless of cell size (i.e. the number of plasmids per cell is unaltered as the growth rate increases, although the plasmid concentration decreases). The exact behaviour of the chromosome as the growth rate changes is unclear, but since faster growing cells possess nested replication forks (Cooper and Helmstetter 1968), the actual number of chromosomal origins must increase as the growth rate increases. However, the increase for completed chromosomes is less than that observed for the cell size, such that the overall concentration of chromosomal DNA decreases. (i.e. The number of chromosomes per cell increases as the growth rate increases, although the actual concentration of chromosomes decreases. Contrast this with plasmids, where the number per cell remains constant, while the concentration decreases.) Examination of experimental data indicates that whereas the plasmid concentration appears to vary over an eight-fold range, that of chromosomal DNA only varies over a two to three-fold range with similar changes in growth rate (Bremer and Dennis 1987, Lin-Chao and Bremer 1986, J. March unpublished results, Stephen Black unpublished results). Thus, measurements of 'plasmids per chromosome equivalent' can be misleading unless the cultures are being grown at essentially identical growth rates.

Although the manner in which chromosomal DNA changes as a whole with changes in growth rate is unclear, the behaviour of certain markers (e.g. the origin of replication or the terminus) can be more accurately modelled (Bremer and Churchward 1977, Chandler and Pritchard 1975). Thus, the ratio of plasmid DNA to a specific marker can be more realistically used as a means of expressing plasmid copy number. This can only be done if an accurate and precise means of estimating the gene dosage of the specific marker exists (such as quantitative hybridisation using a marker-specific probe).

8.1.2 Effect of position in the growth curve on plasmid copy number

It has been discussed above how changes in cell size affect plasmid copy number/concentration. Since cell size varies depending upon the position in the growth cycle, it is important to take this into consideration when measuring copy number. For example, it would be misleading to compare the copy number in an exponentially growing culture with that in a stationary phase culture, since cell sizes would vary considerably. Apart from this, it appears that the rate of plasmid replication varies according to the position in the growth cycle. As a result it is desirable that plasmid-containing cultures be in steady state growth prior to copy number determination. For both ColE1 and ColD derivatives it is clear that the plasmid copy number increases as the culture begins to go into stationary phase (Stueber and Bujard 1982, Frey and Timmis 1985). For ColE1-types at least this appears to be correlated with the rate of cell division (Stephen Black, unpublished results). When a stationary phase culture is innoculated into fresh medium, it undergoes a period of growth during which cell size increases several fold but cell division does not occur. This is a result of the requirement that a minimal volume be attained before septation can begin (Donachie 1968). Plasmid replication appears to behave in a similar fashion; replication does not occur until some time after growth begins, being roughly correlated with the onset of cell division. There then follows a period of steady-state growth during which cell numbers, cell mass, and plasmid numbers increase coordinately and which represents the optimum period for measuring not only plasmid copy number, but for performing enzyme assays etc. The increase in plasmid concentration as the culture enters stationary phase is probably similar to the situation seen with cell division, in which the increase in cell mass slows down but the cells continue to divide for quite some time, resulting in smaller cells. Thus measuring the plasmid concentration at an early stage in the growth cycle would give an under-estimate compared to steady state, while measuring it in early stationary phase would give an over-estimate.

In summary probably the most important aspect of the measurement of plasmid copy number is that cultures be in an identical state with regard to growth rate and position in the growth cycle. Often this is not possible, for example when comparing plasmid copy numbers in a temperature sensitive strain for example, or examining a mutant with a severely reduced growth rate. In most instances it is probably best not to use the ratio 'plasmids per chromosomal equivalent', since the behaviour of the bacterial chromosome varies with changes in cell size. 'Plasmids per cell' or 'plasmid concentration' are better ways of expressing plasmid copy number, since the ratio changes in a more predictable way with changes in cell size.

8.1.3 Methods of measuring plasmid copy number

As was intimated earlier, four general methods of estimating plasmid copy number are available, and a more detailed assessment of these techniques shall now be made.

Enzymatic assay of a plasmid-encoded product: An indirect technique for estimating plasmid copy number is the enzymatic assay of a product encoded by the plasmid of interest. Such a technique is technically simple, and assumes a direct relationship between the amount of plasmid-encoded product and the number of plasmids in the cell. Such an assayable protein is β-lactamase (the product of the ampicillin resistance gene), the activity of which is reportedly linear up to a copy number of 50 plasmids per cell (Neil Willets, pers. comm. to Sullivan 1983). The main advantage of such a technique is rapidity; a large number of samples can effectively be 'batch processed', and use of the system in estimating the plasmid copy number during promoter fusion studies has been made (e.g. Sullivan and Donachie 1984). The main drawback of this approach is that it gives an indirect estimate; the level of β-lactamase activity being taken as representative of the plasmid copy number. Thus factors which affect this measured activity without actually affecting the copy number could give misleading results. Such instances might be affects on transcription of the *amp* gene (either via RNA polymerase or readthrough transcription from a strong promoter on the plasmid), or on the activity of the resultant β-lactamase protein (protein processing/stability or activity for example). Both of these problems were in fact encountered during the course of this work.

Measurement of antibiotic resistance: The principle of this technique is simply that the higher the plasmid copy number, the higher the level of plasmid-encoded detoxifying enzyme synthesised, and therefore the higher the level of antibiotic resistance conferred upon the host. This is probably the most rapid method of

estimating plasmid copy number, and was used extensively during mapping of the *pcn* locus (chapter 7).

However, as was shown in chapter 7, only the measurement of ampicillin resistance levels really lends itself to this approach, since for the majority of antibiotics the level of resistance conferred is decidedly non-linear with respect to gene dosage. In addition, this is a much more indirect measure of plasmid copy number than an enzymatic assay, being two stages removed from the DNA level. Factors which affect the antibiotic resistance level alone would give misleading results (as discussed above). This was found to be a significant problem in a number of instances in this study: for example, over-expression of the *groE* genes was found to reduce ampicillin resistance but not plasmid copy number (section 6.6). However, this approach is certainly of use under certain conditions, although these really require that differences between strains and plasmids be kept to a minimum to reduce the risk of uncontrolled factors. (For example, when mapping the *pcn* locus, strains and their plasmids were identical apart from the *pcn* mutation.)

Curing curves as an estimate of plasmid copy number: The rate of curing of a plasmid from a particular culture can be taken as an estimate of the plasmid copy number in some instances, although again, this is only an indirect estimate. What is really being measured is the *stability* of the plasmid rather than the copy number *per se*, although obviously the former depends on the latter to a certain degree. However, this means that anything which affects plasmid stability while not the actual copy number (such as a partitioning function for example) could give misleading results. Additionally, in many instances such a system can really only be used to estimate relative differences in copy number rather than give absolute values in terms of the number of plasmids per cell. Curing curves, by necessity, demand that the plasmid be unstable to a certain degree: for high copy plasmids such as pBR-derivatives, a large scale reduction in plasmid copy number is probably required before such an approach can be used to estimate copy number. That is, the copy number must be reduced to such an extent that the plasmid becomes inherently unstable in the absence of selection. Curing curves have been used on a number of occasions to give information on plasmid copy number (Meacock and Cohen 1980, Hinchliffe *et al.* 1983), but these have used low copy plasmids which are lost at a high rate. Plasmid loss can therefore be assayed under a realistic time scale. The inaccuracies and time requirements mean that such a technique is probably best not used in isolation as a means of determining copy number, but rather to augment data obtained using other approaches.

Direct measurement of plasmid DNA: Theoretically, direct visualisation of plasmid DNA should be the most accurate means of determining the plasmid copy number in a given culture since it gives a direct measurement of copy number rather than the indirect methods outlined above. The main problem with such an approach involves separating the plasmid DNA away from the contaminating RNA and chromosomal DNA, since the methods used to visualise plasmid DNA (such as Ethidium Bromide staining or radiolabelling) would label these contaminating species at the same time. The widely used alkaline lysis technique of Birnboim and Doly (1979) provides a rapid small-scale partial purification of plasmid DNA which may be viewed directly following agarose gel electrophoresis and ethidium bromide staining. This type of investigation has been used on a variety of occasions to estimate plasmid copy number, with the subsequent densitometric scanning of gel photo negatives allowing a quantitative measurement (Stueber and Bujard 1982, Atlung *et al.* 1985a, Lin-Chao and Bremer 1986). However, such a technique involves at least two precipitation steps during which plasmid DNA might be lost, and large amounts of contaminating nucleic acids remain. Additionally, the degree of lysis of the sample is probably variable. The instant crude lysis technique of Barnes (1977) is better in that the entire cell lysate is loaded onto a gel, but the lack of purification (while reducing plasmid losses) means that quantitation is difficult due to the large amount of contaminating nucleic acids present. Again, quality of lysis is probably variable. Despite these problems, both these techniques have been able to give some indication of the low copy number of plasmids such as pJM32 (section 3.4)

Theoretically then, purification of the plasmid DNA away from contaminating RNA and chromosomal DNA should allow a more quantitative estimate of the amount of plasmid DNA present. In measuring copy number the usual practice is to make use of ethidium bromide-caesium chloride (EtBr-CsCl) isopycnic density centrifugation of a crude cellular lysate to minimise plasmid loss. Such purification makes use of the fact that plasmid DNA is generally isolated as a covalently closed circular (ccc) molecule in contrast to chromosomal DNA which is present as linear fragments. Compared to linear or open circular forms, ccc DNA binds less of the intercalating dye ethidium bromide in such a gradient. As a result, plasmid DNA bands at a higher density than the chromosomal DNA, with the RNA being pelleted in such a gradient. The amount of plasmid DNA relative to chromosomal DNA can then be measured to give an estimate of plasmid copy number (see Womble *et al.* 1977). However, although this technique yields plasmid DNA of high purity, it requires that it be in the ccc state. Any open circular or linear plasmid DNA (as would inevitably be present following cell lysis) would not be detected, so the method probably underestimates the amount of plasmid

DNA present. Cress and Kline (1976) when estimating plasmid copy number by a variety of methods concluded that EtBr-CsCl isopycnic density centrifugation probably seriously underestimated the amount of plasmid DNA present, and questioned the validity of this technique as a quantitative estimate of plasmid copy number. A further drawback of the technique is that it is highly expensive, both in terms of the materials cost and the time involved.

8.2 Introduction to quantitative hybridisation

Quantitative hybridisation as a means of measuring plasmid copy number has the advantage that it is a direct measurment of the amount of plasmid DNA present, but unlike the EtBr-CsCl method described above, essentially all of the plasmid DNA can be detected, whether it be in a ccc, open circular, or linear form. Additionally, since it is specific, problems arising out of contaminating nucleic acid species are for the most part eliminated. Essentially the principle of the technique is that single stranded probe DNA is used to assay the amount of plasmid DNA, one or other of the DNAs being radiolabelled such that recovered radioactivity can be used as a measure of plasmid copy number.

Quantitative hybridisation as a means of estimating the amount of chromosomal DNA in a culture has been used on a variety of occasions (Atlung *et al.* 1987, Kellenberger-Gujard *et al.* 1978, Louarn *et al.* 1974). The use of quantitative hybridisation as a means of measuring plasmid copy number dates back to at least 1973 when it was used by Collins and Pritchard to measure the copy number of an F-prime plasmid. Their technique has subsequently been used by a variety of other workers (Cress and Cline 1976, Frey *et al.* 1979). The technique essentially measures the amount of plasmid DNA by DNA:DNA hybridisation of radiolabelled total *E.coli* DNA containing the plasmid of interest, with total unlabelled DNA from a different bacterial species (*Proteus mirabilis*) containing the same plasmid. Since the level of homology between the DNAs of the two bacterial species is low, the majority of hybridisation will come from the two plasmid DNAs, the absolute level being indicative of the copy number of the plasmid. However, it is obvious that this approach is highly unsatisfactory. The plasmid to be assayed must be compatible in both bacterial species, plus a whole host of controls designed to estimate the level of background hybridisation must be conducted. For low copy number plasmids such hybridisation might totally mask the plasmid:plasmid hybridisation. Additionally, the experimental procedure is time consuming and complex, and offers little flexibility.

A better approach, and the one that has been examined in some detail here, is

to bind unlabelled total DNA from the plasmid-containing strain of interest to nitrocellulose filters, and to then probe to these filters using a purified, plasmid-specific radiolabelled probe. The amount of hybridisation is then proportional to the amount of plasmid DNA in the cell extract (i.e. the plasmid copy number). Such an approach has the advantage that a large number of filters can be prepared and effectively 'batch-processed' with a single radioactive probe, and if needs be, re-used under different conditions.

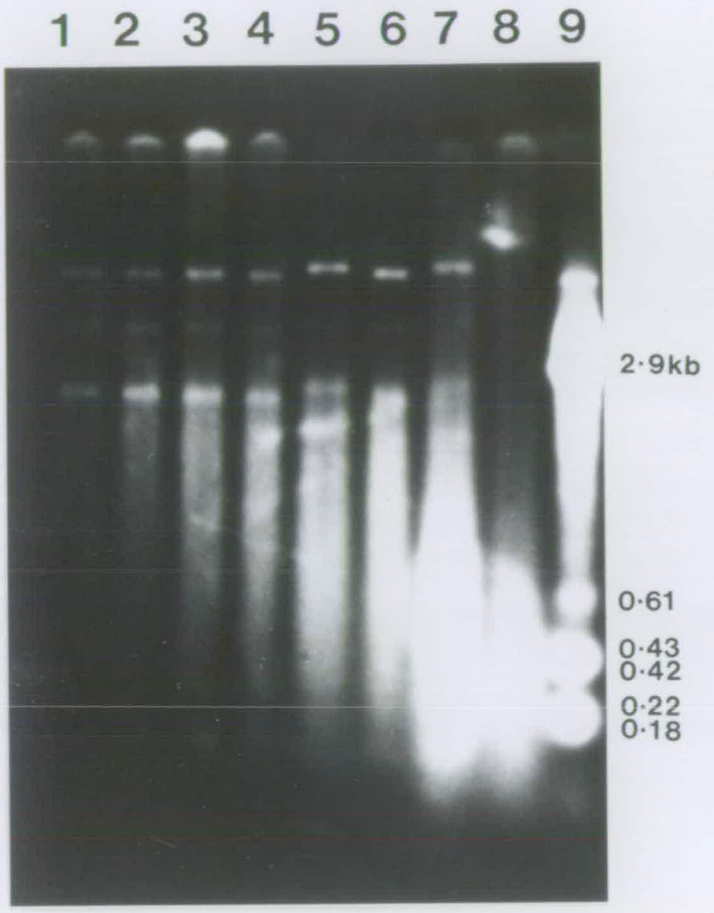
Such a use of quantitative hybridisation as a means of determining plasmid copy number has previously been suggested by Adams and Hatfield (1984); specifically with a view to determining plasmid copy number when performing galactokinase enzyme assays (with the galactokinase gene being carried on a plasmid vector). In their paper they suggested taking a cell extract, sonicating it to disrupt the membranes, and then splitting the sample in two; performing galactokinase assays on one and measuring the copy number (via quantitative hybridisation) on the other. This approach should reduce the possibility of measured differences in galactokinase activity actually being due to undetected changes in plasmid copy number. However, they did not perform an investigation into the factors determining the efficiency and reproducibility of this technique, nor was a detailed experimental protocol produced. It was the objective of the following experiments to provide such information.

8.3 Plasmid yield as a function of sonication time

Sonication as a means of disrupting the cell membrane has the advantage that for the most part enzymes tend to retain their activity, in contrast to the use of lysing agents such as SDS and chloroform which tend to denature proteins. Although simply as a means of determining plasmid copy number *per se* this should not matter, the usefulness of the technique would be enhanced if the method of lysis of the cells were to leave enzymes retaining as much activity as possible should assays be required.

Apart from disrupting the cell membrane and thereby releasing plasmid DNA, sonication also has the effect of shearing the DNA into smaller fragments; the larger the DNA molecule the more likely it is to be broken. As such, the optimum sonication period with which to treat a sample can be seen as being a compromise between the need to properly lyse the cell sample (and thus release as much plasmid DNA as possible), and the requirement that the DNA should not be sheared into too small a fragment size (DNA fragments of less than 150bp in length are not efficiently bound to

FIGURE 8.2 Plasmid yield as a function of sonication time



Autoradiograph of a Southern Blot of a 1% agarose gel

Track 1 C600 pKOC1 (3.6kb plasmid) 1s sonication
Track 2 C600 pKOC1 2s sonication
Track 3 C600 pKOC1 4s sonication
Track 4 C600 pKOC1 6s sonication
Track 5 C600 pKOC1 10s sonication
Track 6 C600 pKOC1 15s sonication
Track 7 C600 pKOC1 30s sonication
Track 8 C600 pKOC1 60s sonication
Track 9 pBR328 HaeII size standards

nitrocellulose membranes; in addition, the stringency of washing required to remove background counts may reduce the level of hybridisation for fragments this small (Thomas, 1980)). Because of this, an investigation into the optimum sonication period with which to treat a sample was therefore conducted.

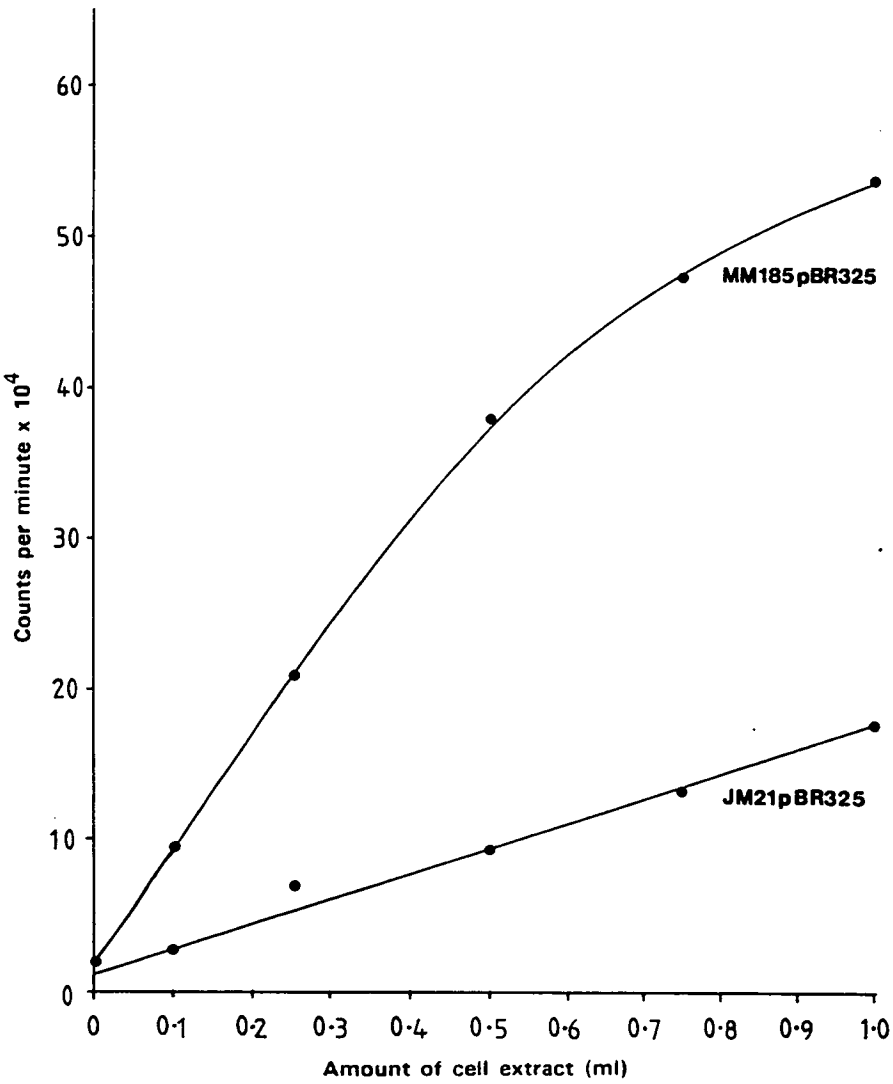
Samples of an exponentially growing plasmid containing strain (C600 pKOC1) were taken and subjected to sonication periods of 1,2,4,6,10,15,30 and 60 seconds using an MSE sonicator at 6μm peak to peak amplitude. Cell debris was removed by centrifugation and the supernatant run out on an agarose gel prior to Southern Transfer and probing with radiolabelled pBR328 (for full experimental details see section 2.8.17). The result of this experiment is shown in figure 8.2. As discussed above, increased sonication times would appear to improve the yield of plasmid DNA whilst concurrently increasing the likelihood that it will be sheared into smaller fragment sizes. Bands corresponding to covalently closed circular, open circular and linear forms can clearly be seen; the amount of covalently closed circular plasmid being dramatically reduced after a 30 second sonication period. On the basis of this experiment a period of 30 seconds would appear to be the optimum, resulting in sheared DNA of a size range of 1-2 kb, whilst still retaining a significant proportion of open circular plasmid. Sonicating the sample for 60 seconds would appear to be too long, resulting in much reduced hybridisation, presumably due to the small size of the sheared plasmid DNA.

8.4 Determination of an experimental technique

On the basis of the above experiment a sonication period of 30 seconds appeared to be the best compromise as regards ensuring efficient lysis of the culture whilst preventing excessive shearing of the plasmid DNA. Gel electrophoresis and subsequent Southern Transfer of the lysed cell sample was obviously capable of visualising the plasmid DNA, and quantitation of the sample would be possible by measuring the band intensity using densitometry. However, such a technique is unnecessarily protracted, and instead it was decided to spot the lysed cell sample directly onto nitrocellulose filters as suggested by Adams and Hatfield (1984).

The main problem regarding quantitative hybridisation was that no indication of the range over which a linear response might occurr was available. For example too little DNA would result in masking by non-specific binding, while too much DNA on the filters could cause saturation such that the response would become non-linear. It was therefore decided to spot varying amounts of the lysate onto filters such that a

FIGURE 8.3 The linearity of the hybridisation signal with changes in the amount of filter-bound DNA



The hybridisation signal obtained with different volumes of total cell extract spotted onto nitrocellulose filters. Linearised pBR325 DNA used as the probe. See text for full details.

graph of the amount of cell extract against radioactivity could be obtained, and data subsequently used from that section in which a linear response could be seen. An example of such a graph can be seen in figure 8.3. This shows the hybridisation of radiolabelled pBR325 probe DNA (100ng in a hybridisation volume of 10ml) to aliquots of total cell extracts of two strains, MM185 pBR325 and JM21 pBR325. Both strains were sampled at the same $O.D._{540}$, and are identical apart from the *pcnB* mutation in

JM21 which reduces the plasmid copy number (see chapter 7). It is clear that much less hybridisation for a given volume of aliquot is seen for this low copy strain. In addition, it can be seen that the hybridisation curve for MM185 pBR325 appears to be reaching saturation point for the largest volume of cell extract. Thus the effective 'plasmids per O.D.' (i.e. the plasmid concentration) can be taken as the amount of hybridisation at a point on the curve where the response is linear, divided by the amount of extract spotted on. From this data it would appear that in a comparative analysis the copy number of pBR325 in JM21 is approximately 25% of that in MM185.

Of course such data is only useful in a comparative analysis rather than in absolute terms; for example it gives no information about absolute numbers of plasmids per cell, nor would it allow comparison with an assay performed on a different day with a probe of a different specific activity. For these purposes an internal standard is required, for example a known quantity of standard plasmid DNA which can be used to enable comparison with data from different assays. This shall be discussed further in section 8.8.

8.5 Determination of the best lysis technique

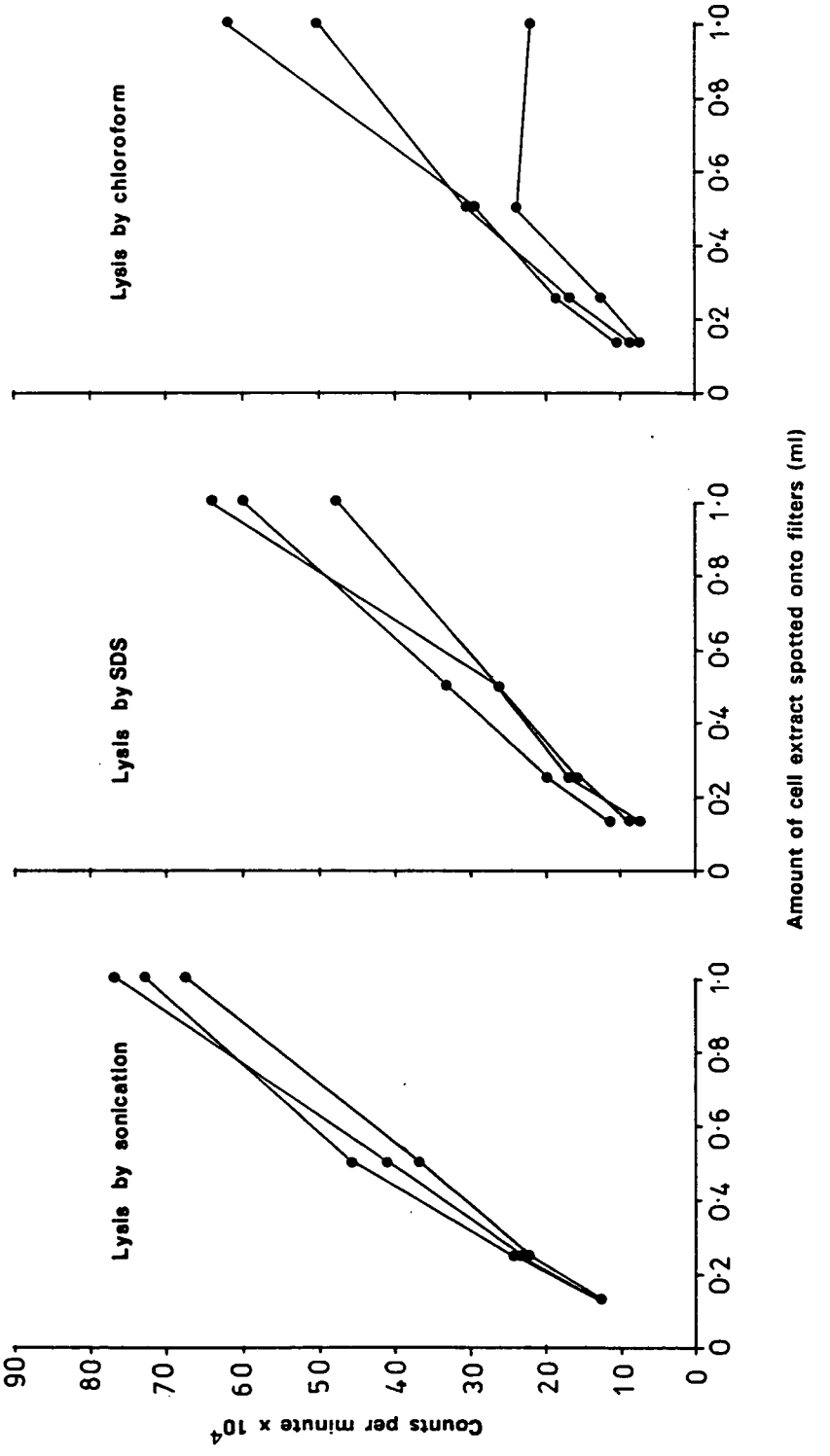
Although sonication as a means of lysing the culture appeared to have the advantage that enzyme denaturation could be minimised by keeping the sample on ice, purely from the viewpoint of measuring plasmid copy number it might not be the most efficient lysis technique. To investigate this it was decided to perform a comparative analysis of three different cell lysis techniques, to see which resulted in the most efficient cell lysis, and to discover which gave the most reproducible results.

An exponentially growing culture of MM185 pBR325 was taken, chilled on ice, pelletted and resuspened in one-tenth of the volume of T.E. 0.6 ml aliquots were taken and treated as follows:-

1. Sonicated for 30 seconds as per usual, or
2. 20µl of chloroform was added and the sample was vortexed for ten seconds, or
3. SDS was added to a final concentration of 1%

All samples were then processed as described in Materials and Methods (section 2.8.16). In addition, each assay was performed in triplicate as an indicator of reproducibility. The results of this experiment are shown in figure 8.4.

FIGURE 8.4 Graphs of the hybridisation signal following different forms of cell lysis



It is clear that sonication appears to be the most efficient technique for lysing the culture. This is both in terms of reproducibility, and in the overall level of hybridisation observed. For this latter point it difficult to know whether the generally higher level of hybridisation following sonication is due to more efficient release of plasmid DNA, or whether shearing of the plasmid DNA into smaller fragments is effectively increasing the total number of plasmid fragments available for hybridisation. However, this should not be a significant problem if all DNA samples are sheared to the same degree. To guard against this, plasmid DNA used as an internal control was also subjected to sonication under the same conditions as the sample.

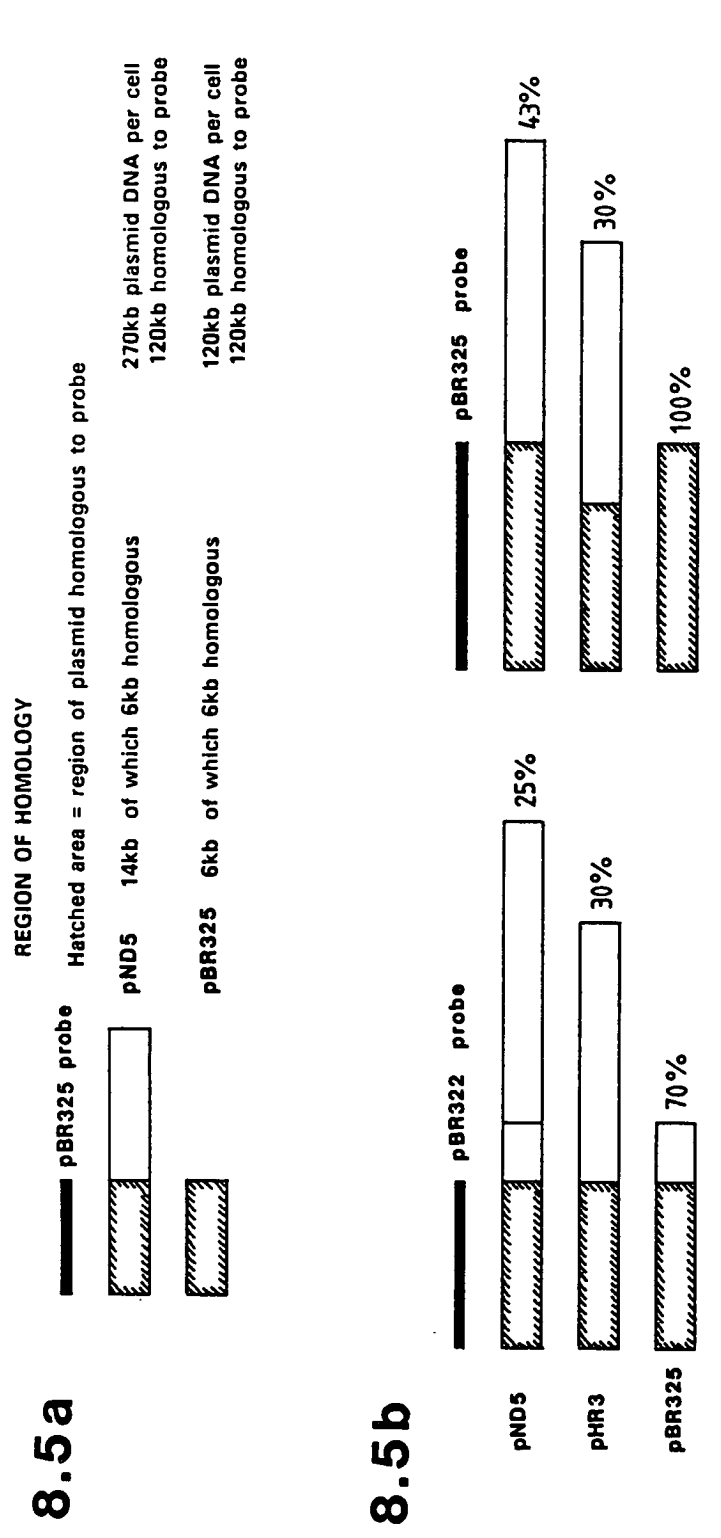
8.6 Is the hybridisation signal proportional to the region of homology?

For the technique to be of more general use it is necessary that the hybridisation signal between probe and plasmid be proportional to the region of homology. This is essential if the copy number of different sized plasmids is to be compared. This is most easily appreciated with the aid of a diagram (figure 8.5a).

Assume the copy number of plasmids pND5 (chapter 3) and pBR325 are being compared, using radiolabelled pBR325 DNA as the probe. Plasmid pND5 contains all of pBR325 (6kb) together with an additional 8kb of non-homologous DNA. Thus in comparing the plasmid copy number between two cells, one containing pND5 and the other pBR325, if both cells have an identical plasmid copy number (for example 20 plasmids per cell), the amount of homologous plasmid DNA will be the same. However, in the pND5 containing cell, there will be 130% extra plasmid DNA due to the non-homologous insert. Thus although the number of 'plasmids per cell' is the same between pBR325 and pND5 containing cells, the amount of plasmid DNA is greater in the latter.

For this relatively straightforward situation no adjustment to hybridisation figures is necessary, since both plasmids have the same amount (i.e. 6kb) of DNA homologous to the probe. However, the situation is more complex when attempting to measure the copy number of a plasmid such as pJM32 (chapter 3). This plasmid only contains 4.4kb of pBR325 DNA, with a further 4.4kb of DNA being non-homologous. Thus in contrast to the situation above, when comparing an identical number of pJM32 and pBR325 plasmids the former will only display 70% of the hybridisation of the latter. Plotting the data graphically, this would mean that an identical copy number should be exhibited as pJM32 displaying a graph of only 70% of the gradient seen for pBR325. Paradoxically enough, since pJM32 is a larger plasmid

FIGURE 8.5 Changes in the proportion of probe-homologous DNA: Theoretical considerations



than pBR325, there will actually be more plasmid DNA present in a pJM32 containing strain when the copy numbers are the same, even though the amount of hybridisation observed will be less.

The choice of probe DNA is important also. The situation described immediately above only arises because the probe DNA is larger than the region of homology in one of the plasmids (i.e. the 4.4kb homologous region of pJM32 is contained wholly within the 6kb pBR325 probe). If, for example, a 3kb probe contained wholly within this 4.4kb pJM32 fragment was used, then both pBR325 and pJM32 would have an identical region of homology with the probe DNA, and an identical copy number would be exhibited by identical hybridisation figures. Thus a judicious choice of probe DNA should allow elimination of corrective factors in correlating hybridisation data between different plasmid DNAs. (i.e. Use of a probe with a region of DNA wholly contained within all of the plasmids being examined.)

On the basis of these theoretical considerations it was decided to examine whether the amount of hybridisation was indeed proportional to the amount of DNA homologous to the probe contained within each plasmid. For this purpose a variety of plasmids, each containing different amounts of vector DNA were spotted onto filters, and the gradient of radioactivity against the amount of DNA was measured. For example, using such data it could then ascertained whether an identical amount of plamsid DNA with 100% homology to the probe would exhibit twice as much radioactivity as one with only 50% homology, as would be expected if the above hypothesis were correct under experimental Figure 8.5b demonstrates the effect that changing the probe DNA has on the expected hybridisation figures. For example, in plasmid pHR3 the homologous region makes up 30% of the total plasmid whether pBR325 or pBR322 is used as a probe, since the vector DNA for this construct is pBR322. However, in the case of pND5, in which the vector DNA is pBR325, the ratio changes from 43% homology (pBR325 probe) to 25% homology (pBR322 probe). The graphs showing the radioactivity counted against the amount of plasmid DNA spotted on the filters were obtained, and the gradients measured. These were then plotted as the measured level of homology against the actual homology, with pBR325 DNA being taken as 100% homologous. In this instance pBR325 was used as the probe DNA; hence the 100% homology. Unfortunately, equivalent data using pBR322 DNA as a probe (in which case pBR325 would only exhibit 70% homology) was not obtained. The plasmid DNAs tested were pBR325 (6kb; 6kb homologous), pPM4000 (12kb; 6kb homologous), pND5 (14kb; 6kb homologous), pHR3 (14.5kb; 4.4kb homologous) and pPM1000 (29kb; 6kb homologous). The graph obtained is shown in figure 8.6. From

this it is clear that a fairly good correlation between the measured and actual homology exists, indicating that such a technique is indeed a valid method of measuring the copy number of different sized plasmids.

FIGURE 8.6 The measured level of homology plotted against the actual level of homology for a variety of plasmid DNAs

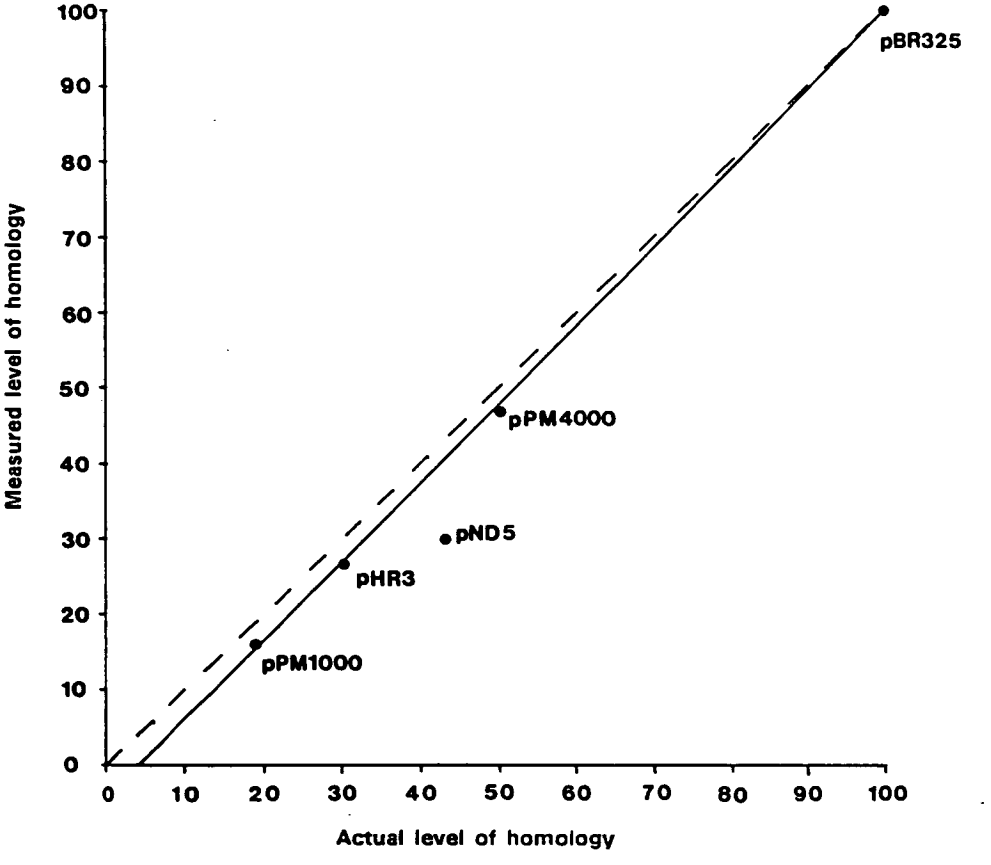


Figure shows gradient of the measured level of homology against the actual level of homology against the probe DNA (pBR325) for each of the plasmid DNAs. Dotted line shows the theoretical line where measured homology exactly equals actual homology.

8.7 Example of the technique in calculating relative plasmid concentration

The calculation of plasmid copy number in strains MM185 and JM21 with a variety of different plasmids is presented below, as an illustration of the use of the technique. These strains are isogenic apart from the *pcnB18* allele carried in JM21, which results in a lowered plasmid copy number for pBR-type plasmids (see chapter 7). The probe DNA in this instance is pBR325, and the plasmids to be measured are

pBR325, pND5 (pBR325 + insert) pHR3 (pBR322 + insert) and pJM32 (deleted version of pBR325 + insert). Plasmid pJM32 was only measured in strain MM185. Each strain was grown for several hours until steady-state growth was achieved, whereupon samples were taken for analysis at an $O.D._{540}$ of 0.35. All strains were grown at 30°C with the exception of the two pND5-containing strains which were grown at 37°C, in L-broth containing ampicillin to maintain selection for the plasmids.

The graphs showing the degree of hybridisation against the amount of cell extract are shown in figure 8.7. Also shown is a standard curve using various amounts of pBR325 DNA, which can be used to calculate the absolute number of plasmids per cell (see section 8.8) or as an internal control to allow comparison with assays performed at a different date. (Note however that if the standard curve is to be used for this purpose then identical hybridisation conditions - i.e. hybridisation volume, amount of probe DNA and stringency of hybridisation - must be used for such comparisons to be valid. Note also that if the standard filters are to be used to estimate the amount of DNA present on the assay filters, then all filters should be hybridised in the same hybridisation bag under the same conditions.) From these graphs the gradient was calculated and the relative levels of hybridisation for each plasmid at 1ml of extract estimated. The calculated 'plasmid copy numbers' (that is, plasmids per O.D. equivalent, or plasmid concentration) are shown in table 8.1, taking into account the amount of DNA homologous to the probe in each instance. In the *pcn*$^+$ strain MM185 it is clear that pBR325 has the highest copy number, with both pND5 and pHR3 being somewhat reduced. The reduction in the *pcnB* strain is also apparent, reducing plasmid copy number to about 25% of the level seen in the wild type strain for both pBR325 and pHR3. In addition, the copy number of pJM32 can be seen to be very low, only about 15% of that of pBR325 in a *pcn*$^+$ strain. Although the copy number of pND5 is reduced in the *pcnB* strain, it appears to be reduced to a lesser extent than pBR325 or pHR3. This may be a result of growth of the strains at a higher temperature, or possibly some feature of the plasmid (such as over-expression of the *groE* genes) is causing this effect.

FIGURE 8.7 Graphs showing the hybridisation signal from different plasmids in MM185 and JM21

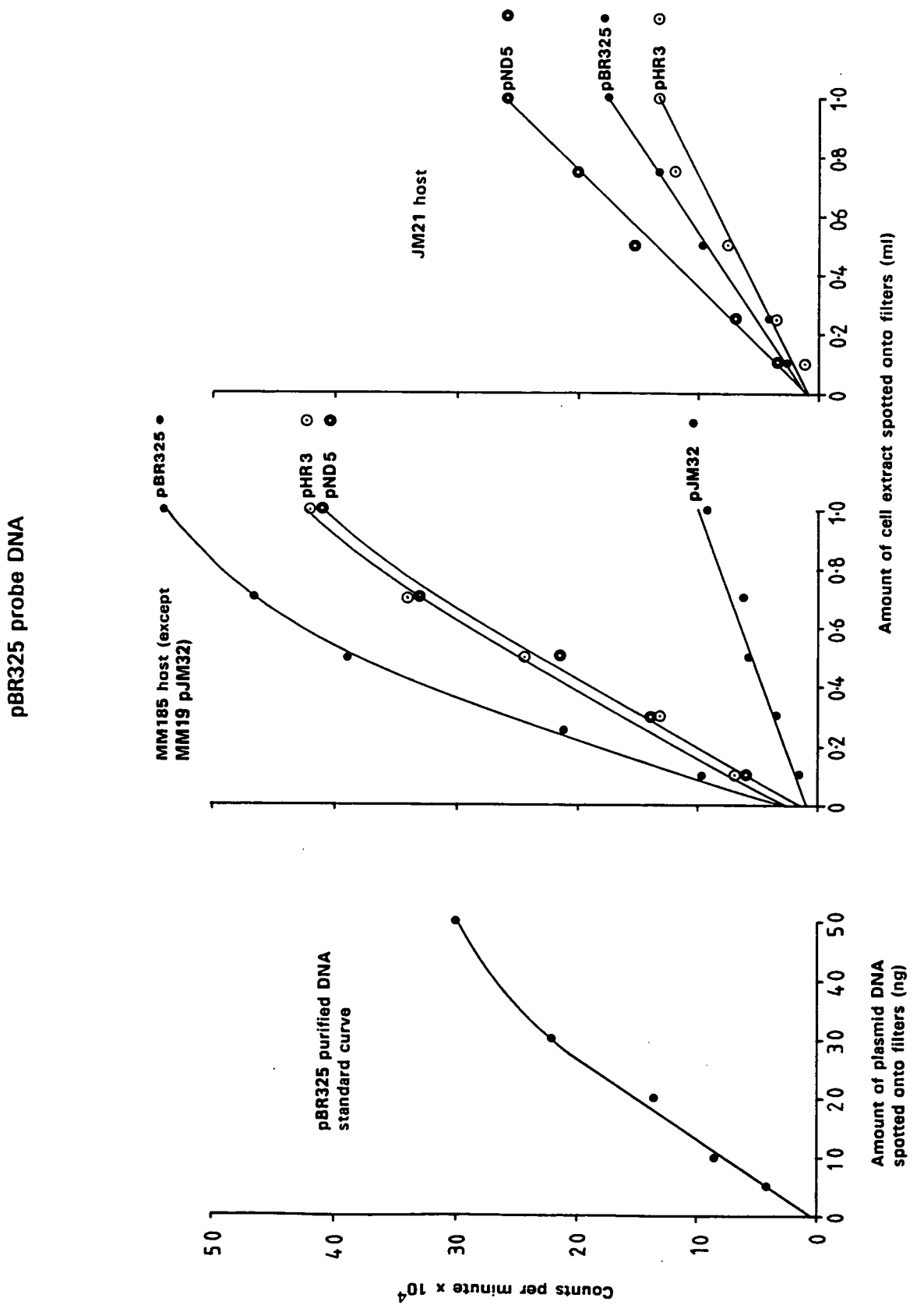


TABLE 8.1 Relative plasmid concentration as measured by quantitative hybridisation

STRAIN	PLASMID	HYBRIDISATION FIGURES	VECTOR:PROBE RATIO[a]	CORRECTED FIGURES[b]	NORMALISED PLASMID CONC.[c]	RATIO JM21:MM185[d]
MM185	pND5	410	1.0	410	0.55	65%
JM21	pND5	265	1.0	265	0.36	
MM185	pBR325	740	1.0	740	1.00	24%
JM21	pBR325	175	1.0	175	0.24	
MM185	pHR3	440	0.73	600	0.81	30%
JM21	pHR3	130	0.73	178	0.24	
MM19	JM32	80	0.73	110	0.15	

a. The ratio of the amount of probe-homologous DNA in the plasmid, to the size of the probe (e.g. for pJM32; 4.4kb pBR325 DNA present; since the probe (pBR325) is 6kb in length, ratio is 4.4/6).

b. Hybridisation figures corrected for the amount of homologous DNA present in the plasmid (e.g. for pJM32 80 x (100/73) = 110).

c. Data normalised to MM18 pBR325 (740) = 1.00

d. The ratio of the copy number of a plasmid in the JM21 background to the copy number in the MM185 background, expressed as a percentage.

8.8 Calculation of the absolute level of plasmids per cell using a standard curve

Apart from allowing a comparison of data from different assays, the standard curve can also be used to give an estimate of the number of plasmid molecules per individual cell if the number of cells in a given volume of culture is known. For MM185 pBR325 this was estimated at 4.4 x 10^8 per ml by performing viable counts after serial dilutions, and from this the plasmid copy number was estimated at c. 35 molecules of pBR325 DNA per cell (figure 8.8). That is, at an $O.D._{540}$ of 0.35, in a mid-log phase steady-state culture of MM185 pBR325 being grown at 30°C with a doubling time of 50 minutes (measured from the growth curves), an individual cell would be expected to contain about 35 copies of pBR325 on average. This is in good agreement with previously published estimates for ColE1-type plasmids (Lin-Chao and Bremer 1986, Muesing *et al.* 1981), and illustrates the validity of the technique. (From the ampicillin resistance curves in figure 7.12 the plasmid copy number of pBR325 can be estimated at c.40 per cell taking a resistance level of 50µg ml^{-1} as being representative of 1 copy of the β-lactamase gene.)

FIGURE 8.8 Calculation of the plasmids per cell figure for MM185 pBR325

From pBR325 purified DNA standard curve (figure 8.7):-

30ng of pBR325 DNA gives a signal of 24 x 10^4 cpm, therefore

1ml of MM185 pBR325 cell extract gives a signal of 75 x 10^4 cpm

equivalent to **100ng** of pBR325 DNA

Number of cells in 1ml of MM185 pBR325 extract = 4.4 x 10^8

Used 6ml; after processing (section 2.8.16) final volume = 5.8ml

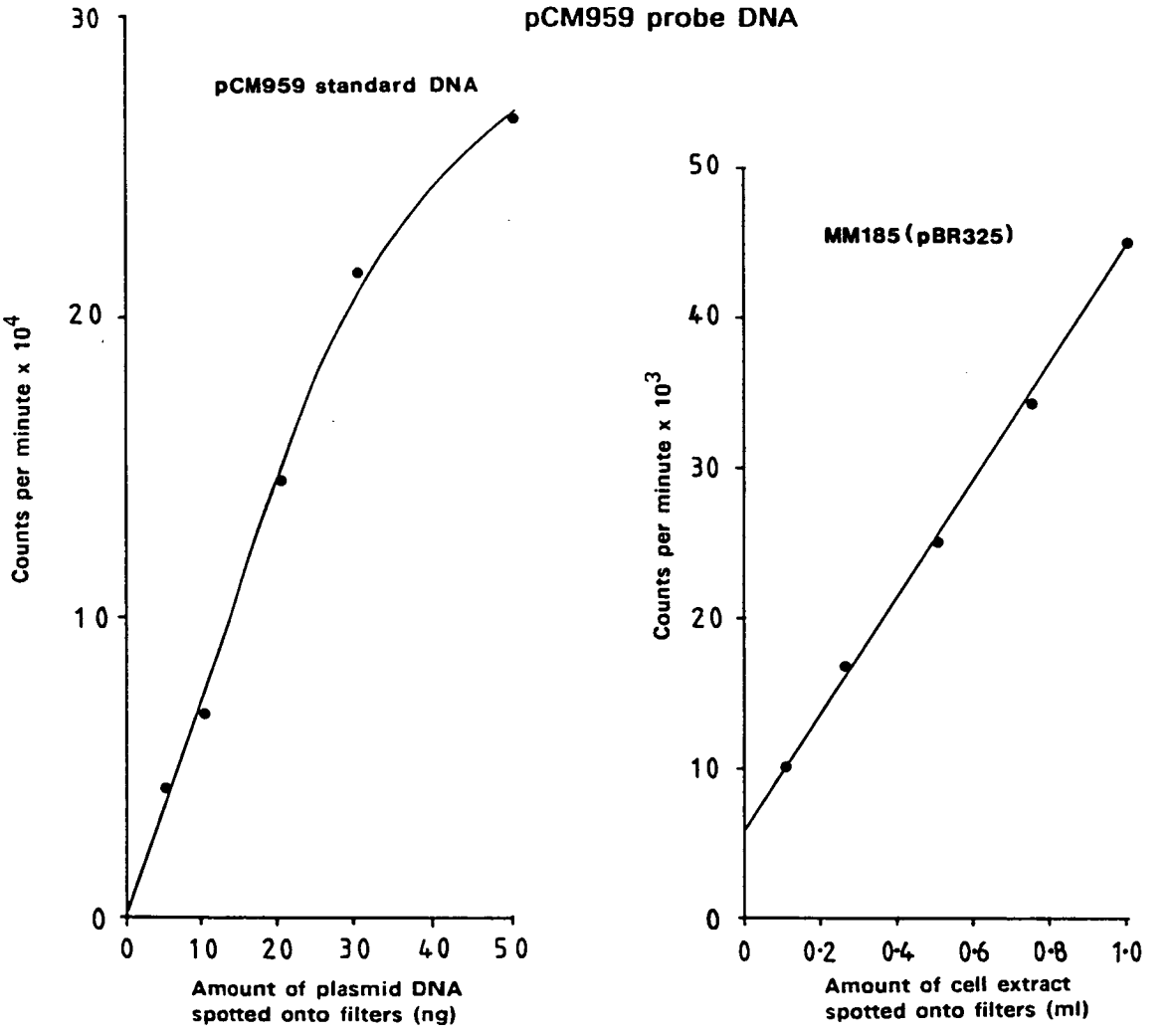
equivalent to **4.6 x 10^8 cells ml^{-1}**

100ng of plasmid DNA = $\frac{100 \times 10^{-9} \times 6 \times 10^{23}}{3.72 \times 10^6}$ = 1.6 x 10^{10} molecules

Therefore **Plasmids per Cell** = $\frac{1.6 \times 10^{10}}{4.6 \times 10^8}$ = **35**

However, it should be remembered that this figure only represents the average number of molecules per cell; it gives no information regarding the range of copy number between different cells. At present however it is not possible to determine the plasmid copy number in an individual cell, and consequently this is probably the best estimate that can be expected.

FIGURE 8.9 Graph showing the level of hybridisation against an origin probe for MM185 pBR325



Purified pCM959 DNA used as the probe. MM185 pBR325 filters were duplicates of those used to produce the graph shown in figure 8.7 (using a pBR325 DNA probe to detect plasmid DNA rather than the origin DNA as in this instance).

FIGURE 8.10 Calculation of the plasmids per origin and origins per cell figure for MM185 pBR325

From pCM959 purified DNA standard curve (figure 8.9):-

30ng of pCM959 DNA gives a signal of 22×10^4 cpm, therefore

1ml of MM185 pBR325 cell extract gives a signal of 45×10^3 cpm

equivalent to **6.1ng** of pCM959 DNA

$$\text{6.1ng of plasmid DNA} = \frac{6.1 \times 10^{-9} \times 6 \times 10^{23}}{2.49 \times 10^6} = 1.48 \times 10^9 \text{ molecules}$$

$$\text{Therefore } \textbf{Origins per Cell} = \frac{1.48 \times 10^9}{4.6 \times 10^8} = \textbf{3.2}$$

$$\text{Therefore } \textbf{Plasmids per Origin} = \frac{1.6 \times 10^{10}}{14.8 \times 10^8} = \textbf{10.8}$$

8.9 Calculation of origins per cell and plasmids per origin figures

The 'plasmids per origin' ratio can also be calculated if the amount of origin DNA in the culture is measured. To do this a duplicate set of filters were probed using purified *oriC* DNA (pCM959; 100ng in a hybridisation volume of 10ml). A standard curve using known amounts of pCM959 DNA was obtained (as per pBR325), and from this the number of origins per cell could be calculated in an analogous manner to that used to calculate the plasmids per cell above. The graphs showing the pCM959 standard curve, and the level of MM185 pBR325 hybridisation to the origin probe are shown in figure 8.9. The calculation of the origins per cell is shown in figure 8.10. From these a figure of 3.2 origins per cell is obtained. That is, in a culture of MM185 pBR325 growing at 30°C with a doubling time of 50 minutes in mid-log phase steady state growth, each cell will on average contain 3.2 origins. This is in fairly good agreement with calculated figures of 2.4 origins per cell at a doubling time of 60 minutes, and 3.4 origins per cell at a doubling time of 40 minutes (Bremer and Dennis 1987), although it should be remembered that these figures refer to *E.coli* B/r growing at 37°C rather than the *E.coli* K12 derivative (30°C) under study here. Additionally, since MM185 carries the *dnaA46ts* mutation, despite the fact that it is being grown at the permissive temperature replication may be affected to a certain

degree even at 30°C. From these figures the plasmid to origin ratio for plasmid pBR325 in strain MM185 is calculated at 10.8 copies plasmid per origin averaged over the culture (calculations shown in figure 8.10.)

8.10 Summary

The results in this chapter concern an investigation into the use of quantitative hybridisation as means of estimating plasmid copy number. On several occasions during the course of the work described in this thesis an accurate measurement of the plasmid copy number was required, and conventional techniques appeared too indirect to be considered suitable under the circumstances (this was especially true when working with *groE* containing plasmids such as pND5 and pJM32).

Quantitative hybridisation is a direct measurement of the plasmid copy number, and therefore should be less susceptible to such effects. Its advantage over other such techniques is that essentially 100% of the plasmid DNA should be detectable, and since a specific probe is being used, problems of contaminating nucleic acids are essentially eliminated. The protocol has made use of purified double-stranded plasmid DNA as a probe, with the filter-bound DNA being in the excess. Under these conditions the level of hybridisation appears proportional to the amount of DNA on the filter, with the response being linear up to about 30ng of homologous DNA per filter (but not necessarily total DNA per filter). However, there appears to be no reason why single stranded DNA should not be equally suitable for use as a probe under similar conditions (for example an M13 insert). Examination of various experimental parameters has suggested that lysis by sonication is the most efficient and reproducible technique of those tried, and that a lysis time of approximately 30 seconds is optimum. It would also appear that the degree of hybridisation detected is proportional to the amount of homologous DNA present in the plasmid: the technique is therefore suitable for comparing the copy numbers of plasmids containing different amounts of vector and/or insert DNA. With no additional information other than the optical density of the culture at the time of sampling, a comparative estimate of plasmid concentration between different cultures can be made. If the number of cells in the sample is known, and a standard curve using known amounts of homologous DNA has been obtained, then the data can also be presented as plasmids per cell. Finally, if a standard curve using a chromosomal marker has been obtained (such as *oriC*), and the molar amount of this marker has been estimated in the culture, then the plasmid copy number can also be expressed as

the ratio of 'plasmids to marker' (e.g. plasmids per origin). Figures obtained using all three approaches have given data comparable with estimates obtained using other techniques, indicating the validity of the approach. Finally, unlike many other commonly used approaches such as EtBr–CsCl density centrifugation, the approach is neither particularly expensive of time or materials, and a large number of samples can be effectively 'batch-processed'.

CHAPTER 9

Summary and Conclusions

9.1 Introduction

The results presented in chapters 3-6 of this thesis concern an investigation into the mechanism by which over-expression of the *groE* genes is able to suppress temperature sensitive mutations in the *dnaA* gene of *Escherichia coli*. Due to the complexity of the available data, each section has included an integral discussion, rather than presenting a discussion of all the experimental results *en masse* in a separate chapter at the end. Instead, this concluding chapter concentrates on summarising the results and conclusions presented earlier, such that an overview of the available information concerning the interactions of the *groE* and *dnaA* gene products is available to the reader.

9.2 Chapter 3: The subcloning of the *groE* genes and an investigation of GroEL protein homologies

The results in this chapter describe the characterisation of a recombinant plasmid, pJM32, originally isolated during an attempt to subclone the *groE* genes out of pND5. Plasmid pJM32 contains the entire promoter region and coding sequence of *groES*, although approximately 10% of the carboxyl terminus of *groEL* is missing. During the construction of the plasmid, the orientation of *groE* transcription with regard to the plasmid origin of replication was reversed. In pJM32, transcription from the strong chloramphenicol and *groE* promoters now reads in tandem towards the replication origin. This is most likely the cause of the reduced copy number observed for this plasmid (only about 15% of that of the vector alone).

Despite the fact that pJM32 is both a low copy plasmid and is missing 10% of the terminal portion of *groEL*, suppression of *dnaAts* mutations at the normally restrictive temperature is possible. This is suprising, since examination of GroE protein levels in a pJM32-containing strain reveals no significant over-production. From this, it was concluded that the truncated GroEL peptide appears to be more efficient at suppressing *dnaAts* than the full size protein.

GroEL possesses a repeated sequence of met-gly residues at the carboxyl terminus. This sequence is absent from pJM32, and the significance of this deletion with regard to the suppression of *dnaAts* was discussed. A computer search of the protein sequence data base revealed that glycine rich sequences are present in a variety of proteins; however, these proteins appear to fall into distinct types. These are:- Keratins, Viral Capsid Prcteins, Cysteine Proteases, Colicins, and Heat Shock Proteins. In additon, individual members of each of these classes exhibit homology to

the main body of the GroEL protein, suggesting a common function or purpose. From these homologies it was postulated that GroEL most likely fulfils a structural role in the cell, particularly with regard to the assembly of large macromolecular structures (such as a viral capsid or an 'initiation complex' -Kornberg 1988, McMacken *et al.* 1987- for example). Why should the truncated GroEL suppress *dnaAts* more efficiently though? The deletion of the glycine rich tail may release GroEL from complex with other proteins perhaps, and thus make it more available for interaction with the DnaAts protein. Alternatively, alteration of the conformation of GroEL by this deletion may make DnaAts a better substrate for suppression. This would strongly argue for some kind of interactive suppression. It was noted that this observation is not without precedent; over-expression of *groE* is also able to suppress temperature sensitive mutations in the *ams* gene of *E.coli*, and again, suppression appears to be more efficient with a truncated GroEL protein. Finally, these observations were discussed in the light of the recently observed homology (Hemmingsen *et al.* 1988) between GroEL and the Rubisco subunit-binding protein. This homology strongly implicates GroEL as playing a role in the post-translational assembly of macromolecular structures. Again, this suggests that the mechanism of suppression of *dnaAts* by over-expression of *groE* may be due to the assembly and/or maintenance of an initiation complex at *oriC*. The fact that DNA replication is affected in *groE* mutants (Tilly *et al.* 1981, Wada and Itikawa 1984) would strongly implicate GroE as playing a role in the process. However, against this model is the observation that none of the initiation mutants tested (with the exception of *dnaAts*) are suppressed by over-production of GroE (although a more detailed investigation into this should perhaps be conducted).

9.3 Chapter 4: An investigation into possible mechanisms of suppression of *dnaAts* mutations by over-expression of the *groE* genes

This chapter mainly concerns a genetic analysis of the possible mechanisms of suppression. Since *dnaAamb* mutants are not suppressed, it was concluded that suppression is not bypass in nature, and that it requires the presence of the DnaAts protein. It was noted that the pattern of allele specificity of suppression by over-expression of *groE* exactly matches that of some secondary mutations in *rpoB* (Atlung 1984) which are able to suppress *dnaAts*. Additionally, a clustering of suppressed alleles in the central portion of the gene was noted. This supports the idea that suppression may be due to a specific interaction between GroE and DnaA, a hypothesis further strengthened by the additional clustering seen with those alleles suppressible to 42°C against the majority of alleles which are only suppressible up to 40°C. This implies that it is the *location* of the point mutation within the protein

-rather than the particular *type*- which determines suppression, again arguing the case for interactive suppression.

The effect of over-production of GroE on the expression from the *dnaA* promoter was examined. This was intended as a preliminary investigation of whether suppression is mediated via over-production of the mutant DnaAts protein. This was conducted by examining the β-galactosidase levels using a DnaA-β-galactosidase translational fusion (Braun *et al.* 1985) under a variety of different conditions. This clearly showed that suppression is not due to an increase in expression from the *dnaA* promoter, although this result does not preclude the possibility that suppression may result from increasing the DnaAts protein level by some other means (effect on *dnaAts* mRNA levels, translational efficiency, DnaAts protein stability for example). Additionally, these experiments demonstrated that over-expression of *groE* is able to suppress the autorepression function of DnaA as well as its initiation function. An examination of whether GroE might affect protein degradation was undertaken using a β-galactosidase-*P.falciparum* fusion protein. However, this gave inconclusive results, and suggested that a direct examination of DnaAts protein levels would be the best method of testing whether suppression is mediated via this route. Might over-expression of *groE* prevent induction of the heat shock response (of which proteases are a component; Goff *et al.* 1984), and thus protect DnaAts from degradation? It was noted that cells over-expressing *groE* appear to be abnormally sensitive to heat shock. However, such cultures appear to filament normally, suggesting that heat shock is indeed being induced, and that the increased sensitivity to heat may simply be a result of 'GroE poisoning'.

The phenomenon of 'cold sensitivity' (reduced growth at 30°C for certain *dnaAts* alleles in combination with over-expression of *groE*) was discussed. Certain aspects of cold sensitivity suggest that it may be a direct consequence of the mechanism of suppression. These are:- all cold sensitive alleles can be suppressed; cold sensitivity is allele specific, and the locational clustering of the most cold sensitive alleles within the *dnaA* gene suggests that cold sensitivity may be due to some specific interaction; the pattern of allele specificity is very similar to the pattern of '*oriC*' sensitivity (that is, reduced growth when transformed with an *oriC* plasmid) seen for *dnaAts* alleles. However, evidence was presented which suggests that cold sensitivity is not a direct consequence of the process of suppression (most notably its occurrence in an *oriC* delete strain over-expressing *groE*). The allele specificity of cold sensitivity due to over-production of GroE is the same as that seen with *dnaAts*/*dnaA*$^+$ merodiploids, suggesting that informational suppression (leading to

both mutant and non-mutant forms of the DnaA protein) may be taking place. However, as was comprehensively discussed in section 4.7, this is unlikely to be the mechanism of suppression.

9.4 Chapter 5: The cloning of *dnaA*, the construction of a β-galactosidase-DnaA fusion protein, and the raising of DnaA antisera

The raising of anti-DnaA sera was undertaken in order to enable a more direct investigation into the mechanism of suppression. This was particularly important with regard to an examination of DnaAts protein levels. Translational fusion studies had indicated that DnaAts levels are not increasd as a result of increased expression from P*dnaA*. However, they could be increased by a variety of other means (effects on mRNA stability, translational efficiency or protein degradation for example). In addition, it was hoped that DnaA antiserum could be used to look for possible interactions between DnaA and other cellular proteins (for example, can DnaA be co-precipitated with GroE?).

To raise anti-DnaA sera it was decided to construct a β-galactosidase-DnaA fusion protein, to purify this protein (by one or more of several well-characterised techniques making use of its uniquely large size and β-galactosidase activity), and use it for subsequent immunization. The cloning of *dnaA* was first undertaken. This was eventually successfully accomplished, although in the process a variety of 'partial' high copy suppressors of *dnaAts* were obtained. These partial suppressors fell into several different classes, some of which could be used to transform *dnaAts* strains directly to temperature resistance, others of which required pre-growth at 30°C. Further characterisation of these suppressors was not undertaken. It was noted that plasmid pHR3 (containing the *rpoBC* genes; Newman and Hayward 1980) appears to be able to partially suppress the *ts* phenotype of 10 different *dnaAts* alleles tested. That is, it does not appear to increase the maximum temperature at which growth is possible, although cultures containing pHR3 grow markedly better than controls which lack this plasmid at the higher temperature. These results were taken as being indicative of a large number of cellular interactions for the DnaA protein.

The β-galactosidase-DnaA fusion protein encoded by plasmid pJM88 results in filamentation and cell death if induced at 37°C or above, although induction at lower temperatures does not markedly reduce cellular growth or viability. The protein appears to be about 170kd in size, and it is not clear if it possesses DnaA activity.

Purified fusion protein was used to obtain DnaA antiserum. Western Blotting

using purified DnaA protein (obtained from E. Whale) demonstrated that the serum possessed anti-DnaA activity, as well as anti-β-galactosidase activity. The antiserum was used to show that less than 1000 molecules of DnaA protein are present in an individual cell, an estimate in line with those of other researchers (Sakakibara and Yuasa 1982, Sekimizu *et al.* 1988). Unfortunately however, the low quantities of DnaA in the cell prevented use of the antiserum to visualise cellular DnaA protein under experimental conditions. Further purification of the antiserum should help in this respect.

Finally, antibodies reacting to an *E.coli* protein of about 60kd were found in the sera from three different rabbits. These antibodies were present in the pre-immune sera at a very much lower level, and it appears that they are reacting to GroEL. It is unclear if GroEL was present as a contaminating protein during the immunization step (which would imply an association between GroEL and DnaA), or whether this is due to a cross-reacting antibody to some other protein (as is suggested by the presence of these antibodies in the pre-immune sera, although at a very much lower level). In support of this latter hypothesis, GroEL-like proteins appear to be highly antigenic and present in a variety of bacterial species (Young *et al.* 1988), so it is conceivable that the contaminating antibodies are not against the *Escherichia coli* GroE protein itself, but are the result of an earlier challenge by a similar protein type.

9.5 Chapter 6: The phenotypic effects of over-expression of the *groE* genes

The presence of the *groE* genes in high copy number results in a variety of phenotypic side effects to the cell, additional to the ability to suppress *dnaAts* mutations. The extent of these effects appears to be temperature dependent, suggesting that they are indeed due to over-expression of *groE*, rather than of another gene contained within the insert DNA (expression of *groE* being temperature-dependent; Herendeen *et al.* 1979). This is further suggested by the nature of these effects.

Over-production of GroE appears to result in a reduced resistance to the antibiotic nalidixic acid in an already resistant strain (by virtue of the *gyrA* mutation). Excess GroE results in an initial reduction in the growth rate. After a period of time, the culture appears to become 'adapted' and the growth rate returns to near normal. The period of adaption depends upon the extent of GroE over-production; the more GroE, the longer the lag. Cultures pre-grown in the presence of nalidixic acid do not show this extended lag when diluted back into fresh nalidixic acid-containing medium.

That this is not simply due to 'GroE poisoning' (nalidixic acid being known to induce *groE* expression; Krueger and Walker 1984) can be seen from the absence of this lag following ethanol shock or UV irradiation (which also induce *groE* expression). As was discussed in section 6.5, taken together, the data suggests some kind of interaction between GroE and gyrase, implicating the former in the process of DNA replication, although why cultures over-producing GroE should eventually become 'adapted' to high levels of nalidixic acid remains unclear. Since gyrase plays an important role in the initiation of DNA replication (as well as in the elongation stage; Drlica 1984), this may represent an interaction between GroE and gyrase in this process, possibly an interaction in which DnaA may play a part also. An examination of whether *groE* (either mutant alleles or over-expression) is able to suppress temperature sensitive mutations in *gyrA* is suggested by these findings.

Apart from reducing host cell resistance to nalidixic acid, over-expression of *groE* also appears to result in a reduced resistance to ampicillin (in a strain resistant by virtue of β-lactamase production). This agrees with, and possibly provides an explanation for, data by Kuriki (1987), in which he showed that induction of the heat shock response causes a reduction in the production of β-lactamase, although since the β-lactamase mRNA levels appeared unchanged he concluded that this reduction is at the translational level. Since β-lactamase is made in an inactive precursor form and subsequently processed to the active form, this implies that GroE may have a role in post-translational processing. However, the levels of the various forms of the β-lactamase proteins appear to be unchanged in minicells over-expressing *groE*, suggesting that the reduced resistance may be due to some other mechanism. An examination using a variety of different membrane damaging agents led to the conclusion that excess GroE does not lead to a general increase in host cell sensitivity to membrane damaging agents.

Finally, in contrast to the aforementioned phenotypic effects (in which over-expression of *groE* results in an increased sensitivity to a particular agent), host cell resistance to the antibiotic streptomycin (in a strain resistant by virtue of an *rpsL* mutant allele) is increased following over-expression of *groE*. Since GroE is reportedly associated with ribosomes (Neidhardt *et al.* 1981), the increased resistance may be the result of excess GroE physically 'blocking' streptomycin from interacting with the RpsL protein. Alternatively, this increased resistance may be a reflection of GroE over-producing strains being better able to cope with the multitude of aberrant proteins likely to result from streptomycin induced mis-translation.

9.6 Conclusions

Taking the available information regarding *dnaA* and *groE* together, what can be said about the most likely mechanism by which suppression of *dnaAts* by over-expression of *groE* is occurring?

Suppression is not bypass in nature, since the presence of the DnaAts protein is required (sections 4.2 and 4.7). Over-expression of the *dnaAts* gene does not occur in suppressed strains, indicating that suppression is not mediated via this route (sections 4.5 and 4.7). What is the possibility that the level of DnaAts protein may be increased by some other means? It was hoped that DnaA antiserum would help in answering this question, by allowing a measurement of DnaAts protein levels in suppressed and non-suppressed strains. However, even though this did not prove possible to accomplish, suppression is unlikely to be the result of an increase in the level of the DnaAts protein. Work by Sakakibara and Yuasa (1982), in which they examined DnaA46ts protein levels using 2D gels, showed that no degradation occurred following thermal inactivation of the DnaA46ts protein. In addition, their work, and later studies by Sekimizu *et al.* (1988), suggests that the cell normally maintains an excess of DnaA protein, over and above that required to participate in the initiation reaction. This would suggest that merely increasing the amount of the DnaAts protein is unlike to overcome its inability to participate in the initiation reaction. In fact, early work conducted using *dnaAts* mutations (Hansen and Rasmussen 1977, Orr *et al.* 1978) suggested that degradation of the DnaAts protein was unlikely to be occurring following thermal inactivation. These results showed that a *dnaAts* mutant acquired excess initiation capacity when maintained at the restrictive temperature. This suggests the accumulation of thermally-denatured DnaAts protein at the restrictive temperature rather than its degradation. That is, it is the *activity* rather than the *amount* of the DnaAts protein which is limiting under such conditions. The fact that initiation is then able to proceed following a temperature shift down (in which *de novo* protein synthesis is prevented by the addition of chloramphenicol), shows that sufficient DnaAts protein is available for initiation to occur.

Most of the available data would point to suppression being the result of a direct interaction between DnaA and GroE (or possibly via an intermediary protein). If suppression is the result of a more general mechanism (for example the 'repair' of the *ts* protein; protection from degradation; informational suppression -discussed in section 4.7-; an increase in the amount of the DnaAts protein), then a mulitude of other temperature sensitive mutations would be expected to be suppressed. This is not seen. Instead, the pattern of allele specificity of suppression would suggest that it

is the location of a mutation within DnaA -rather than the particular type- which determines whether it is suppressible. The clustering of alleles which are suppressible up to the higher temperatures would further support this. In addition, the fact that a truncated GroEL protein appears to be more efficient at suppressing *dnaAts* would add further weight to this hypothesis, suggesting that the alteration to GroEL has rendered it better able to interact with DnaAts. The known intracellular function of GroEL, as a protein involved in the assembly of macromolecular structures would suggest that suppression may be the result of an interaction between GroEL and DnaAts during initiation, possibly in the assembly of an 'initiation complex'. The likelihood that GroEL and gyrase appear to interact in some manner (section 6.3), together with the reduced DNA replication seen in *groE* mutant strains (Wada and Itikawa 1984), would support this hypothesis. Finally, the observed homologies between GroEL and a variety of protein types has suggested not only that GroEL may be intimately involved in the process of macromolecular assembly, but that the mechanisms of such assembly may be highly conserved from simple prokaryotic viruses right through to complex multicellular organisms.

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APPENDIX I

Published Work

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MGG

A DNA fragment containing the *groE* genes can suppress mutations in the *Escherichia coli dnaA* gene

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Summary. An 8.2 kb fragment of *E. coli* chromosomal DNA, when cloned in increased copy number, suppresses the *dnaA46* mutation, and an abundant protein of about 68 kd (60 kd when measured by us), encoded by the fragment, is essential for the suppression (Takeda and Hirota 1982). Mapping experiments show that the fragment originates from the 94 min region of the chromosome. It encodes several proteins but only one abundant polypeptide of the correct size, the product of the *groEL* gene. Suppression by the fragment is allele specific; those mutations which map to the centre of the gene are suppressed. Other initiation mutants including *dnaA203*, *dnaA204*, *dnaA508*, *dnaAam*, *dnaC*, *dnaP* and *dnaB252* are not suppressed. Most suppressed strains are cold-sensitive suggesting an interaction between the mutant proteins (or their genes) and the suppressing protein or proteins.

Key words: *groE* – *dnaA* – Replication initiation – *E. coli*

Introduction

Initiation of DNA replication at the chromosomal replication origin, oriC, is dependent, both in vivo and in vitro, upon the product of the *dnaA* gene (Hirota et al. 1968; Fuller and Kornberg 1983). Temperature-sensitive mutants of this gene have been invaluable in helping to elucidate its role in replication and allowed the important distinction betwen initiation and elongation of replication to be made at an early date. Temperature-resistant derivatives of *dnaA*(Ts) mutants can easily be isolated and the mutations conferring temperature-resistance mapped (Wechsler and Zdzienicka 1975; Atlung 1981). Although some are back mutants in the *dnaA* gene itself, most serve to define a number of different, potentially suppressing, genes. At least some of these genes seem to specify proteins capable of interacting with the *dnaA* product during initiation (Bagdasarian et al. 1977; Atlung 1984).

During an attempt to clone the *dnaA* gene, we isolated a λ phage containing a chromosomal DNA insert capable of reversing the Ts phenotype of a *dnaA46* (Hirota et al. 1968) mutant. The insert proved to contain, not the *dnaA* gene, but a suppressing DNA fragment which, since it did not originate from mutated DNA, most likely exerted its effect as a result of increased production of a protein encoded by it. Takeda and Hirota (1982) and Projan and Wechsler (1981) have reported the isolation of DNA fragments which can suppress *dnaA*(Ts) mutants when cloned in high copy number. One of the fragments isolated by Takeda and Hirota (and which contains the suppressor which they term *sda*) seems identical to our fragment. They clearly demonstrated that a protein encoded by the fragment, and with a molecular weight which they measured as 68 kd, is essential for suppression. They did not, however, identify or map the gene encoding the protein. We report here the chromosomal mapping of the suppressing fragment and the probable identity of the 68kd suppressing product with that of the *groEL* (*mopA*) gene. We have analysed its pattern of suppression and find that only those temperature-sensitive alleles of *dnaA* which are located in the middle of the *dnaA* gene and which are suppressible by the *rpoB902* mutation (Atlung 1984; Hansen et al. 1984) are suppressed by the fragment. The mutations *dnaA203*, *dnaA204* and *dnaA508*, located to the left and right of the suppressible alleles, are not suppressed.

A related study of *dnaA* suppression by a *groE*-containing DNA fragment is described by Fayet et al. (1986).

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Methods and materials

Bacteria, phage and plasmids. The bacterial strains used are described in Table 1. λNM616 was obtained from N. Murray (Mileham et al. 1980) and λ425 from A. Wright (Schaus et al. 1981). Plasmids used were pBR325 (Bolivar 1978) and pSC101 (obtained from P. Meacock).

Media and growth conditions. Luria Broth (described in Masters 1970) was used for non-selective growth of liquid cultures and solidified with 1.5% agar in plates. Tetracycline (10 μg/ml), chloramphenicol (30 μg/ml) or ampicillin (50 μg/ml) was added to selective medium as required. Minimal medium (VB) was that of Vogel and Bonner (1956). Trimethoprim was added to minimal plates at 50 μg/ml when needed. Frd^+ transductants were selected and scored using the medium described by Guest and Nice (1978). Plates were incubated in anaerobic jars and Gas-Paks (BBL) used to generate an atmosphere of H_2-CO_2.

Table 1. Bacterial strains

Strain	Genotype/relevant genotype	Derivation/source/ reference
A. *DnaA* mutants and strains used for cloning and expression		
C600	*thr leuB6 thi supE44 lacY1 fhuA21*	Appleyard (1954)
ED419	*thi leu* del*(lac-proB) supE42 dnaA46*	derived from LC343 Bird et al. (1976)
DS410	*thi gal min rpsL*	Dougan and Sherratt (1977)
MM18	*argG6 asnA31 asnB32 hisG1 leuB6 metB1 pyrE gal-6 lac xyl-7 supE44 uhp bglR fhuA2 gyrA rpsL tsx*	derived from MM7 Masters et al. (1984)
MM20	MM18 *polAam*	P1.CM5649 (W.S. Kelley)
JM101	del*(lac-pro) supE thi/F' traD36 proAB lac-18 lacZdelM15*	
JW393	*leu-19 pro-19 trp-25 his-47 thyA59 arg-28 ilv deoB23 lac-11 gal-11* Spc^R *dnaA204*	J. Wechsler
JW397	*metE70 thyA36 deoC2 malB45 rha-5 lacZ53 strA151 dnaA5*	J. Wechsler
JW402	*his trp* del*(lacX74)* Spc^R *rpsE dnaA508 zib-501::*Tn*10*	J. Wechsler
WM1026	*lac supD thi trp* Val^R *ilv dnaA46*	W. Messer
WM1029	as 1026 but *dnaA167*	W. Messer
WM1032	as 1026 but *dnaA508*	W. Messer
WM1152	*lac* IqL8 Val^R *dnaA204*	W. Messer
NS387	del*(lac-proA) trpam his thi supF81*(Ts) *rpsL tsx tna::*tn*10 dnaA311am*	A. Wright (Schaus et al. 1981)
NS388	as NS387 but *dnaA366am bglR*	A. Wright
MM181	MM18 *asnA*$^+$ *dnaA167*	P1.WM1029
MM182	MM18 *asnA*$^+$ *dnaA5*	P1.LC905 (L. Caro)
MM183	MM18 *asnA*$^+$ *dnaA204*	P1.WM1152
MM184	MM18 *asnA*$^+$ *dnaA508*	P1.WM1032
MM185	MM18 *asnA*$^+$ *dnaA46*	P1.WM1026
MM186	MM18 *asnA*$^+$ *dnaA203*	P1.CM748 (Hansen et al. 1984)
MM187	MM18 *asnA*$^+$ *dnaA602*	P1.CM2733 (Hansen et al. 1984)
MM188	MM18 *asnA*$^+$ *dnaA601*	P1.CM2735 (Hansen et al. (1984)
MM189	MM18 *asnA*$^+$ *dnaA604*	P1.CM2738 (Hansen et al. 1984)
MM190	MM18 *asnA*$^+$ *dnaA606*	P1.CM2740 (Hansen et al. 1984)
B. Strains used only in mapping, complementation or suppression tests (with relevant genotype)		
AB259	HfrH Str^S [a]	Jacob and Wollman (1956)
ED3029	Hfr Str^S [a]	Masters (1975)
ED3062	Hfr Str^S [a]	as ED3029 except for point of insertion of F
MM303	*ilv arg metB*	Masters (1977)
MM304	*rha*	Masters (1977)
χ478	*metE*	Masters (1977)
M5000	*glnA*	CGSC[b] Mayer et al. (1975)
AB2569	*metA*	CGSC
H1175	*purA argI*	CGSC Glansdorff (1967)
M2508	*melA*	CGSC Schmitt (1968)
JRG820	*melA*	J. Guest
JRG780	*frd*	J. Guest (Lambden and Guest 1976)
	groE	B. Hohn
PC1	*dnaC1*	CGSC Carl (1970); Wechsler and Gross (1971)
PC2	*dnaC2*	
KY2750	*dnaP18*	Wada and Yura (1974)
RS162	*dnaB252*	CGSC
AB1157	*thr argH*	

[a] For origin of transfer see Fig. 1
[b] Strains labelled CGSC were obtained from B. Bachmann and are part of the *E. coli* Genetic Stock Center Collection

Genetic methods; biochemical methods used in DNA and phage preparation. P1 lysate preparation and transduction were carried out as previously described (Masters 1970). Phage λ was prepared by plate lysates or, when larger quantities were required, by UV induction as described by Wilson et al. (1977). Small-scale preparation of plasmids for screening and checking strains was by the method of Birnboim and Doly (1979). Bulk preparation on CsCl-ethidium bromide gradients was by the method of Maniatis et al. (1982) except that the chloramphenicol amplification step was omitted. Restriction of DNA, agarose gel electrophoresis and transformation were as described by Maniatis et al. (1982).

Cloning. 300 ng of C600 genomic DNA and 600 ng of λNM616 were digested with *Eco*RI and ligated in 10 µl using the conditions described by Maniatis et al. (1982). The ligated DNA was introduced into the Lac$^-$ indicator strain JM101 after being packaged in vitro (Scherer et al. 1981). Thirty percent of the 9000 plaque forming units obtained were of the recombinant type. The library was amplified by preparing a plate lysate on JM101. To reclone the *sdaA* insert into pBR325 1 µg of *Eco*RI digested λ*sdaA* was mixed with 1 µg of *Eco*RI digested pBR325 and ligated in a volume of 10 µl. Fifteen chloramphenicol-sensitive recombinant plasmids were screened; one of these contained the 8.2 kb *sdaA* fragment.

Minicell preparation; labelling, electrophoresis and autoradiography of proteins. Minicells were produced essentially by the method described by Reeve (1979). Overnight broth cultures of plasmid-containing strain DS410, in which antibiotic selection had been maintained, were enriched for mini-cells by centrifuging at 900 *g* for 15 min before proceeding with the Reeve procedure. Frozen aliquots of minicells (equivalent to 1 ml of cells of $OD_{600}=0.2$) were thawed, rinsed and resuspended in 100 µl of M9-glucose. Eight µl of 25% Difco Methionine Assay mix containing 20 µCi of S^{35}-methionine were added and the mixture incubated for 20 min at 20° C. The reaction mixture was then diluted to 1 ml with unlabelled methionine (in minimal medium) to give 0.4 mg/ml methionine, and incubated for a further 10 min before being placed on ice. The labelled minicells were pelleted, washed in 0.5 M Tris-HCl (pH 6.8) and resuspended in 20 µl of loading buffer (Laemmli 1970). This was incubated at 37° C for 60 min before loading onto a 14%–20% gradient SDS polyacrylamide gel. The gel was prepared and electrophoresis carried out as described by Laemmli (1970). The proteins were stained with Coomassie brilliant blue by gently shaking the gel in 0.1% (w/v) Coomassie blue, 9% (v/v) acetic acid, 45% (v/v) ethanol in distilled water for 45′ at 37° C. Excess stain was removed by two 30′ washes in 5% (v/v) ethanol, 7% (v/v) acetic acid under the same conditions. To prevent cracking of the gel, it was soaked in 2% (v/v) glycerol, 10% ethanol at 37° C for 30′ prior to drying. Gels were dried under vacuum at 80° C and autoradiograms made by directly exposing Dupont Cronex 4 film.

Results

Isolation and characterization of λsdaA

λ*sdaA* is a recombinant phage which can promote the growth, at 40° C, of *dnaA46* mutant strains lysogenized with

it. It was constructed from *E. coli* C600 chromosomal and λNM616 (Mileham et al. 1980) DNAs by digesting the DNAs with *Eco*RI, ligating and packaging in vitro. λNM616 contains an inserted *lac* fragment and recombinants can be identified as forming Lac$^-$ plaques. Phage capable of transducing the *dnaA46*(Ts) strain ED419 to temperature-resistance were isolated by spotting the library (it contained approximately 2600 primary recombinants) onto a lawn of ED419 and incubating at 40° C. Clusters of temperature resistant (Tr) colonies appeared in each spot. Ten separate colonies were tested and seven found to produce phage which could, in their turn, induce temperature-resistant growth. These seven temperature-resistant colonies, plus three similar ones isolated from the same library, were purified and the phage they carried analyzed; each contained an 8.2 Kb *Eco*RI insert. Two of the phages contained an additional insert (of 4.0 and 4.4 Kb respectively), indicating that not all the suppressing phage isolated are clonally related. Neither the *dnaA* gene [now know to be interrupted by an *Eco*RI site (Hansen et al. 1982)], nor any part of it, is contained on an 8.2 Kb *Eco*RI fragment. Thus the fragment isolated must suppress rather than complement the *dnaA* mutation. This was confirmed by showing that the 8.2 kb fragment failed to hybridize to λ425 (Schaus et al. 1981), a hybrid phage which carries the *dnaA* gene (data not shown). Further restriction analysis, with the enzymes *Bgl*II, *Bam*HI, *Hpa*I, *Hin*dIII, *Kpn*I, *Pst*I and *Xho*I confirmed that the fragment is almost certainly identical to that isolated by Takeda and Hirota (1982) in pYT47 and containing the suppressing locus which they term *sda*.

Both the symbols *das* (*d*na*A* *s*uppressor) and *sda* (*s*uppressor of *d*na*A*) have been used to denote suppressors of *dnaA*. We would like to suggest that *das* be reserved for suppressing chromosomal mutations such as those described by Atlung (1981) and that *sda* be used for cloned suppressors such as those described by Takeda and Hirota (1982) and Projan and Wechsler (1981). We suggest that the suppressor cloned in pYT47 and which also forms the subject of this paper be called *sdaA*.

Previous non-mutant suppressors of *dnaA*, including *sdaA*, were isolated after cloning in high copy number vectors. Our identification of *sdaA* in λ seemed to imply that it can suppress in low copy number. This is surprising and suggests either that our cloned fragment contains a mutation indigenous to C600 or that it encodes a product which is overexpressed in the λ environment. That C600 does not contain a *dnaA* suppressor was shown by cotransducing *dnaA46* with a neighbouring marker into C600 and showing that the anticipated fraction of progeny were temperature-sensitive. The marker we used was Tn*7* which confers trimethoprim resistance and has a favoured insertion site that is 50% cotransducible with *dnaA* (Barth et al. 1976). The transposon was introduced by P1 transduction into a *dnaA46* strain and a P1 lysate prepared on a trimethoprim-resistant temperature-sensitive transductant. This was used to transduce C600 to trimethoprim-resistance. As anticipated, 50% of the C600 progeny were temperature-sensitive, indicating that C600 does not contain an indigenous suppressing mutation. The suppressed phenotype of λ*sdaA* lysogens of *dnaA*(Ts) strains is thus probably due to increased expression of the suppressing genes. This is unlikely to result from enhanced transcription due to an active upstream λ promoter, as, firstly, the relevant promoters are not active in the lysogenic state and secondly, suppression still occurs when the fragment is recloned in the opposite orientation (data not shown). An explanation for the suppressed phenotype has been provided by Fayet et al. (1986) who have independently isolated a phage similar to λ*sdaA*. They find that only strains which are multiply lysogenized with the phage are suppressed. This may explain our observation that of 20 λ*sdaA* lysogens of *dnaA46* strains isolated at 30° C only 3 were temperature-resistant, although all could produce suppressing phage.

Mapping of sdaA

To facilitate the chromosomal mapping of *sdaA*, we recloned the insert into the *Eco*RI site of the high copy number plasmid pBR325 (Bolivar 1978). The resultant plasmid, pND5 (Fig. 1) was then transformed into a number of Hfr strains. Plasmids containing cloned chromosomal DNA are presumed to undergo frequent unstable recombination events with the homologous chromosomal DNA which enables them to be transferred effectively during conjugation, a process termed "conduction" by Yamada and Hirota (1982). We found that pND5 was transferred by ED3062 and ED3029 (Table 2) but not by AB259, indicating that the DNA homologous to it is located between 83 and 97 min on the standard map.

To further map *sdaA*, P1 transduction was carried out using as donors strains in which pND5 had been integrated into the chromosome adjacent to *sdaA*, thus enabling us to select plasmid-borne drug resistance as a marker in transductional crosses (Greener and Hill 1980; Silver and Wickner 1983). To achieve integration the *polA* strain MM20 was transformed with pND5 DNA and Tet^{r}Amp^r transformants selected. Since pND5 is a *polA* dependent replicon, it cannot survive as a plasmid in a PolA$^-$ host (Kingsbury and Helinski 1970). However, it can be expected to integrate by recombination at the site of homology (i.e. near the chromosomal *sda* locus) and thus be passively maintained. Although recovery of transformants is reduced greatly when integration is required, it is not observed unless homology is present (Table 3), indicating that the transformants recovered are almost certainly homologous recombinants in which the plasmid has been integrated at the chromosomal *sdaA* locus. In order to locate the site of integration precisely, strains with genetic markers between 83 and 97 min were transduced with P1 lysates prepared on the *polA* pND5 strain and wild-type phenotype and drug resistance selected separately and simultaneously. Since the recipients are *polA*$^+$ and since *Col*E1 related replicons do not appear to be maintainable in the integrated state in *polA*$^+$ cells (Yamaguchi and Tomizawa 1980), antibiotic resistance in the progeny will be plasmid-borne while cotransduced chromosomal markers may either be plasmid borne or recombined into the chromosome. Thus, although the occurrence of cotransduction indicates that the marker in question must be within 2 min of *sdaA*, the actual cotransduction frequencies observed in this unconventional cross cannot necessarily be used as a simple measure of genetic distance.

Our initial experiments showed no cotransduction (at least 100 single transductants were scored in cases where double transductants were not obtained) between drug resistance and *asnA* (84.2 min), *ilv* (84.6), *met*E (85.5), *gln*A (86.7), *rha* (87.7), *metB* (89), *arg* (89.5), *metA* (90.5) or *arg* (96.6) but ca. 30% cotransduction was obtained with *pur*A

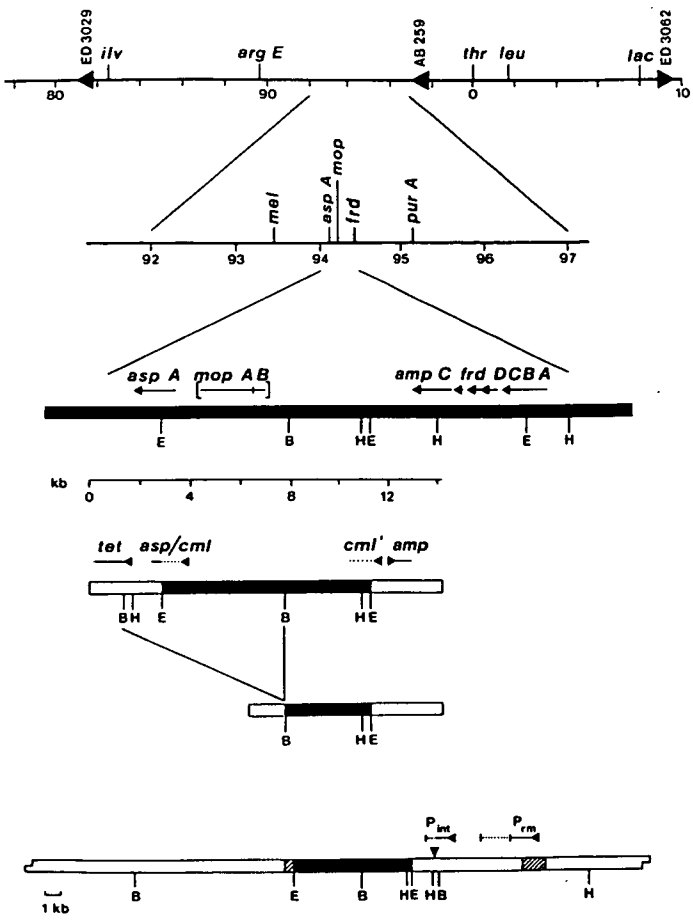

Fig. 1 A–C. Genetic map showing the location of the *sdaA* fragment and physical maps of pND5 and *λsdaA*. A Origin and direction of transfer of Hfr strains used to locate *sdaA*. B Location of genetic markers with which *sdaA* can be cotransduced (Bachmann 1983). C Restriction map showing positions and transcription of neighbouring genes (redrawn from Guest et al. 1984). *E* represents *Eco*RI restriction sites *B: Bam*HI and *H: Hin*dIII. *Arrows* above pND5 and *λsdaA* show positions of known promoters and the directions in which they are transcribed. The *dotted* transcript at the right of pND5 could code for a possible fusion protein that would include the proximal portion of the chloramphenicol-resistance gene. Asp/cml represents a possible fusion protein whose transcript would originate at the *aspA* promoter (Takegi et al. 1985). The drawing of *λsdaA*, in which the *vertical arrow* represents *att*, shows that transcription from the promoters active in the lysogenic state would be separated from the cloned insert at attachment. The *hatched areas* in *λsdaA* represent non-λ DNA

Table 2. Hfr conduction of plasmids

Donor	Chromosomal marker and time of entry	No. of Arg^+ or Thr^+ progeny/ 0.1 ml	No. of Tet^R progeny/ 0.1 ml	Ratio $\times 10^3$
ED3029 (pND5)	*argH* (7 min)	3×10^5	860	2.8
ED3029 (pBR325)	*argH*	7×10^5	20	0.028
ED3062 (pND5)	*thr* (10 min)	0.95×10^5	689	7.3
ED3062 (pBR325)	*thr*	2.7×10^6	0	0
AB259 (pND5)	*thr* (1 min)	1.2×10^5	4	0.033
AB259 (pBR325)	*thr*	8×10^4	0	0

Exponentially growing cultures ($OD_{540} = 0.5$) of male and females (AB1157) were mixed in a ratio of 10:1 and incubated without aeration for 60 min. Dilutions of vortexed cultures were plated on selective minimal and LB tetracycline (10 μg/ml), each containing 200 μg/ml of streptomycin as counterselection

Table 3. Transformation of *polA* and *polA*$^+$ strains

Recipient	Transforming DNA	Transformants per μg of DNA
MM18 (*polA*$^+$)	pND5	1.5×10^5
	pBR325	2.4×10^6
	pPM30	1.7×10^6
MM20 (*polA*)	pND5	1304
	pBR325	0
	pPM30	4×10^5

Transformation mixtures prepared as described were incubated 45 min at 37° C before diluting and plating on selective media, tetracycline (5 μg/ml) for pND5 or pBR325 and ampicillin (50 μg/ml) for pPM30. pPM30 is derived from pSC101 (Meacock and Cohen 1980) and replicates independently of *polA*

(95). The absence of cotransduction with *argI* suggested that *sdaA* is located on the *melA* (93.4) side of *purA* and cotransduction of pND5 and *melA* was subsequently demonstrated (Table 4). Guest et al. (1984) report that a fragment of the correct size and restriction pattern is located at 94.2 min. It is adjacent to *frd* and contains the *groE* (*mop*) genes. Cotransduction of antibiotic resistance and *frd* occurred, although the instability of Tet^r in Frd^+ trans-

Table 4. Transductional mapping of *sdaA*

Cross	Recipient	Selected marker	No. of transductants		Cotransduction (%)			Expected[a]
					Scoring single transductants		Simultaneous selection	or previously reported
1	H1175	PurA$^+$	1225		29		0.7	*purA-groE*
		Tet^R	1025		29		0.7	25% (1)
		PurA$^+$Tet^R	7					
2	H1175	PurA$^+$	118		33		29	
		Amp^R	326				10	
		PurA$^+$Amp^R	34					
3, 4	M2508	MelA$^+$	56	101	0	0	0	*melA-groE*
		Tet^R	280	287	3	9		
		MelA$^+$Tet^R	0					24% (2)
5	JRG820	MelA$^+$	129		0		5	
		Tet^R	545		7		1	
		MelA$^+$Tet^R	6					
6	M2508	MelA$^+$	231		0		0	
		Amp^R	1160		1.4		0	
		MelA$^+$Tet^R	0					
7	JRG780	Frd$^+$	387		13,18[b]		81	*frd-groE*
		Tet^R	1885		17		17	73% (2)
		Frd$^+$Tet^R	315					

[a] Based on (1) Wu (1966) equation and map location (Bachmann 1983) or (2) Guest and Nice (1978). Transductions were carried out as described in Methods

[b] Duplicate determinations

ductants made an accurate estimate of cotransduction frequency difficult. We have no explanation for this instability which also is evident when MelA$^+$ is selected.

To establish the identity of the fragment we had cloned and that containing the *groE* genes, we sought to demonstrate complementation of *groE*$^-$ by *λsdaA*. This was straightforward as *groE*$^-$ hosts, although unable to plate *λ* phages, can plate phages carrying the cloned *groE* genes (Georgopoulos and Hohn 1978; Hendrix and Tsui 1978). *λsdaA* was able to make plaques on the *groE* host although λNM616 and λ425 could not. We thus conclude that the *sdaA* fragment can complement *groE* mutants and that since it maps to the correct position it is the 8.2 kb fragment between *frd* and *asp*.

Is sdaA the groE gene?

An 8.2 kb fragment is large enough to code for approximately 240 kd of protein. Takeda and Hirota (1982) reported that the fragment codes for a 68 kd and a 15 kd protein and that the presence of the larger of these, in its wild-type form, is necessary for suppressing activity. A protein of about this size has been identified as the product of the *groEL (mop)* gene (Georgopoulos and Hohn 1978; Hendrix and Tsui 1978). Since the published gels do not allow the unequivocal identification of proteins specified by the 8.2 kb insert, other than the two encoded by the *groE* genes, we attempted to identify others which might be concerned with suppression. pND5 was transformed into DS410, a minicell producing strain, and plasmid encoded proteins labelled with S^{35}-methionine in purified minicells. The proteins produced were separated on a polyacrylamide gel and an autoradiogram prepared (Fig. 2). The *groE* proteins are very strongly expressed and can be seen as prominent bands not only on the stained minicell gels but even on those of plasmid containing whole cells. In addition to the *groE* polypeptides others with molecular weights of 38 kd, 32 kd, 18 kd, 13.5 kd and two smaller ones are intensely labelled with S^{35} and are specific to the insert. Polypeptides of approximately these sizes, except for the 38 kd polypeptide, were identified by Guest et al. (1984) as amongst those coded by the (12.5 kb) chromosomal fragment overlapping the insert in pND5 and cloned in pGS73.

To further define individual coding regions within the cloned fragment, pJM18 (Fig. 1) was constructed, tested for suppressing activity and expressed in minicells (Fig. 2D). MM185 (pJM18) does not grow at 40° C or 42° C indicating, in agreement with Takeda and Hirota (1982), that the proteins encoded on this plasmid cannot suppress *dnaA46*. Minicells containing pJM18 produce the 32 kd and 38 kd polypeptides, demonstrating that these products are encoded on the right-hand end of the pND5 insert (and that the 38 kd polypeptide is at least partially encoded or expressed from sequences within the *Hin*dIII-*Eco*RI segment absent from pGS73) but fail to produce the 18 kd or either of the *groE* polypeptides. The left-hand portion of pND5, when cloned separately, continues to suppress (data not shown). Thus, although enhanced production of the 38 kd and 32 kd polypeptides is not required for suppression, that of *groES* and *groEL* remains correlated with suppressing activity.

The proteins we have identified account for 76% of

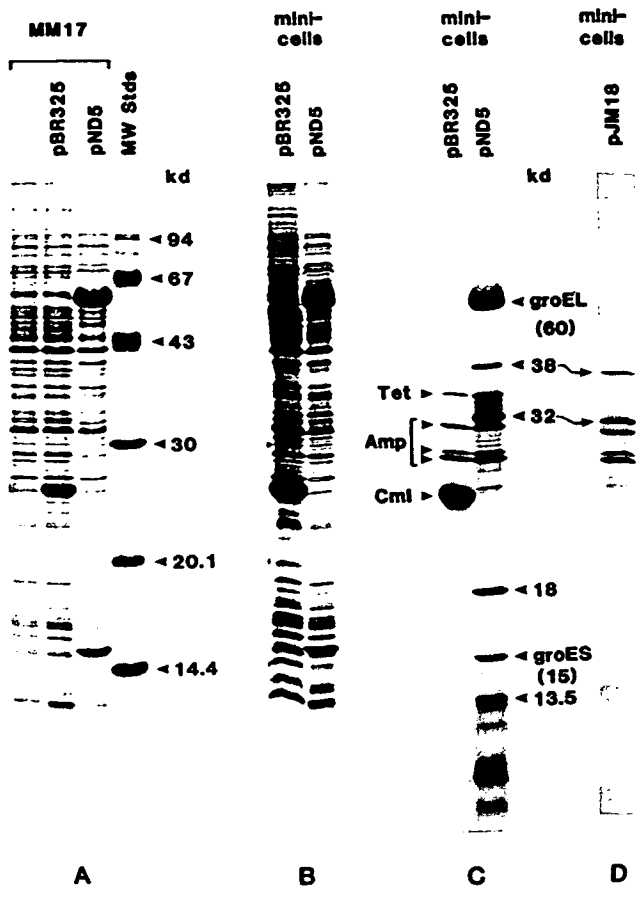

Fig. 2A–D. Production of proteins encoded by pND5. **A** Overproduction of plasmid encoded proteins in whole cells. MM17(MM18*recA*) and plasmid containing derivatives were grown overnight in broth with appropriate anbibiotic selection. The equivalent of 1 ml of cells at $OD_{600}=0.2$ was solubilized and loaded onto the gel (see Methods). Proteins were stained with Coomassie blue. Bands corresponding to the chloramphenicol (pBR325), *groEL* and *groES* (pND5) proteins are clearly visible. **B** Plasmid coded proteins in minicells: Coomassie-blue stained cellular proteins. Minicells were prepared from DS410 containing pBR325 or pND5, labelled with S^{35}, extracted and run on gels as described. The chloramphenicol and *groE* proteins are identifiable as particularly dark bands. **C** Autoradiogram of **B**. All above tracks are from the same gel and should therefore be comparable except that **C** was very slightly magnified relative to the others during printing. **D** Autoradiogram of minicells prepared from pJM18 and labelled as described above

the coding capacity of the insert in pND5 leaving unassigned coding capacity equivalent to no more than 56 kd of protein. Although the insert is large enough to encode a second large protein in addition to the *groEL* product, there is no evidence that such a protein is made.

Thus the abundant 68 kd (60 kd on our gels) protein identified by Takeda and Hirota (1982) as essential to suppression is almost certainly the *groEL* product. Whether one or more of the smaller proteins encoded by this region is also essential for suppression remains to be determined.

Suppression by sdaA

There are several mechanisms by which suppression can occur. Bypass suppression results when the product of the

Table 5. Suppression by *sdaA*

Strain[a]	Mutation	Suppression by		Complementation by *λdnaA*$^+$ [c]
		pND5[b]	*λsdaA*[c]	
MM182	*dnaA5*	+	+[d]	+
MM185	*dnaA46*	+	+	+
MM181	*dnaA167*	+	+	+
MM183	*dnaA204*	−	−	+
MM184	*dnaA508*	−	−	+
MM186	*dnaA203*	−	−	+
MM187	*dnaA602*	+[e]	+	+
MM188	*dnaA601*	+[e]	+[d]	+
MM189	*dnaA604*	+[e]	+[d]	+
MM190	*dnaA606*	+	+	+
NS387	*dnaA311am*	−		
NS388	*dnaA366am*	−		
PC1	*dnaC1*		−	
PC2	*dnaC2*		−	
RS162	*dnaB252*		−	
KY2750	*dnaP18*		−	

[a] Each of the *dnaA* mutant strains listed in Table 1 was tested for suppression by *sdaA*. Results for each *dnaA* allele were the same irrespective of the genetic background of the strain tested
[b] Growth of pND5 transformants when streaked at 42° C. pBR325 transformants were used as controls in all cases
[c] Growth of colonies within phage spots on lawns of test strain at 42° C. All strains were confluently lysed at 30° C. *λdnaA*$^+$ is λ425 of Schaus et al. (1981)
[d] At 40° C; these strains are not suppressed by *λsdaA* at 42° C
[e] At 40° C; these strains are not suppressed by pND5 at 42° C

suppressing gene can substitute for the defective protein or bring into action an alternative pathway which does not require it. In such cases nonsense mutants are suppressed as well as those in which an altered protein is synthesized. To test whether this is the mechanism by which *sdaA* acts we transformed NS387 and NS388 with pND5. These strains contain, respectively, the nonsense mutants *dnaA311* and *dnaA366* suppressed at low temperature by the conditional suppressor *supF81* (Schaus et al. 1981). The parent strain is Ts for growth due to failure to synthesize a *dnaA* product at 42° C and remains so when transformed with pND5 indicating that *sdaA* does not bypass the need for a *dnaA* product.

It has been reported (quoted in Atlung 1984) that the overproduction of the *dnaA*(Ts) mutant protein coded by four of the alleles used in this study (amongst others) will allow growth at 42° C. If *sdaA* suppression works by increasing the amount of *dnaA*(Ts) protein produced (e.g. by interfering with *dnaA* autoregulation (Braun et al. 1985) then it could be anticipated that each of these Ts alleles would be suppressed. We tested ten Ts alleles of *dnaA* to see whether they would be suppressed by pND5. In order to obtain comparable results, each allele was transferred by cotransduction with *asnA*$^+$, into MM18. Temperature sensitive transductants were transformed with pND5 to Tet at various temperatures. The presence of the plasmid was confirmed by DNA preparation and restriction analysis and suppression tests performed. Only seven of the ten alleles tested were suppressed; the other three were not. Similar results were obtained when *λsdaA* was spotted onto lawns of the strains at 42° C (see Table 5). These results suggest

Table 6. Growth of suppressed and unsuppressed *dnaA* mutants

Strain	*dnaA* allele	Growth rate					
		30° C		37° C		42° C	
		Colony[a] diameter (mm)	Liquid[b]	Colony[a] diameter (mm)	Liquid[a]	Colony[a] diameter (mm)	Liquid[b]
MM18 (pND5)	*dnaA*$^+$	0.8	46, 58	1.5	28, 33	1.5	24
MM18 (pBR325)	*dnaA*$^+$	0.8	48, 58	1.5	28, 33	1.5	24
MM181 (pND5)	*167*	1.0		1.3		1.5	
MM181 (pBR325)	*167*	1.3		1.5		0	
MM182 (pND5)	5	<0.1	190	0.1–0.3	52	1.3	90
MM182 (pBR325)	*5*	1.0	55	0	62[c]	0	68[c]
MM183 (pND5)	*204*	0.7		1.3		0	28
MM183 (pNR325)	*204*	1.0		1.5		0	
MM184 (pND5)	*508*	0.7		1.0		0	
MM184 (pBR325)	*508*	1.0		1.5		0	
MM185 (pND5)	*46*	0.1–0.3	73	1.0	45	1.5	38
MM185 (pBR325)	*46*	1.0	47	0	45[c] 100	0	45[c]
MM186 (pND5)	*203*	1.0		1.0		0	
MM186 (pBR325)	*203*	1.0		1.5		0	
MM187 (pND5)	*602*	0		0.3		1.3[d]	
MM187 (pBR325)	*602*	1.0		0		0	
MM188 (pND5)	*601*	0		0.3		1.3[d]	
MM188 (pBR325)	*601*	1.0		0		0	
MM189 (pND5)	*604*	0		0.3		1.3[d]	29
MM189 (pBR325)	*604*	1.0		0		0	
MM190 (pND5)	*606*	0		0.1		1.3[d]	
MM190 (pBR325)	*606*	1.0		0.5		0	

[a] On LB tetracycline (5 μg/ml), measured after overnight growth (ca. 18 h)
[b] Doubling time (in min) of OD_{540} of cultures grown on LB tetracycline
[c] Initial growth rates; these cultures soon filament, and growth rate rapidly decreases
[d] At 40° C; growth rate is reduced at 42° C

a specific interaction between the product(s) of *sdaA* and that of the mutants suppressed.

We obtained further evidence of a specific interaction between suppressible mutant *dnaA* products and *sdaA*. The growth rate at permissive temperature of six of the seven suppressible alleles is reduced in the presence of pND5 (Table 6). Four of these strains fail to grow at all at 30°. This was first noted as reduced colony size when the *dnaA5* and *dnaA46*(pND5) strains were plated at 30°, and confirmed as reduced growth rate in liquid medium for these strains. The severity of this temperature sensitivity varies amongst the suppressed strains, and does not occur at all in the *dnaA167* strains, in the unsuppressed strains or in *dnaA*$^+$ strains. Since cold-sensitivity occurs only in mutants which are suppressed at high temperature, it seems likely that the interaction between the *sdaA* and *dnaA*(Ts) products is responsible for both phenotypes.

Discussion

We have cloned a fragment of DNA from the *E. coli* chromosome that is able to suppress certain temperature-sensitive alleles of the *dnaA* gene. This fragment contains the *groEL* gene, the product of which is greatly overproduced in the suppressed strains. Since Takeda and Hirota (1982) showed that a protein of about the size of the *groEL* product is required for suppression to occur and since no other polypeptide of this size is identifiable as coded by the suppressing fragment, it seems certain that the *groEL* product is the suppressing protein they identified.

The mechanism by which suppression occurs remains obscure. We have shown that *dnaAam* mutants are not suppressed by *sdaA*, indicating that the presence of a suppressible *dnaA* mutant product is required for suppression to occur; that is, *sdaA* does not substitute for, or eliminate the need for, a *dnaA* gene product. Furthermore we find that not all presumed missense mutants are suppressed by *sdaA*. (This differs from the conclusion of Takeda and Hirota (1982) who found that each of the seven *dnaA* alleles they tested was suppressed by pYT47. They did not, however, include the alleles we found not to be suppressed among those they tested; thus our results and theirs are not in disagreement). The allele-specificity of *sdaA* suppression is reminiscent of *dnaA* suppression by *rpoB* alleles (Atlung 1984) and it is worth noting that, within the set of alleles we tested, suppressibility is confined to those mutations that can be suppressed by the *rpoB902* mutation and which code for proteins which are reversibly heat inactivated (Hansen et al. 1984). Also, the alleles we find to be suppressible are located in the centre of the mapped portion of the gene (Hansen et al. 1984). The three that are not suppressed are at the left and right extremes. Taken togeth-

er these observations seem to suggest that the central portion of the *dnaA* gene specifies a functional unit of the protein.

Overproduction of mutant *dnaA* protein has been reported to lead to suppression of the Ts phenotype (Atlung 1984). Amongst the alleles reported as suppressible in this manner are *dnaA203* and *dnaA204*, alleles we find *not* to be suppressed by *sdaA*. Thus it seems unlikely, although not excluded, that *sdaA* suppresses by simply increasing the expression of the mutant alleles. It clearly remains necessary to measure the levels of *dnaA* protein produced in suppressed and unsuppressed mutant strains.

The *groE* product has been reported to be associated with ribosomes (Neidhardt et al. 1981). This introduces the possibility that the suppression we observe is informational, i.e. that excess *groE* protein somehow results in the synthesis of non-mutant as well as mutant *dnaA* product. Allele specificity would be consistent with this explanation. In support of this idea is the observation that strains bearing alleles which, in combination with pND5, result in cold sensitivity are also cold-sensitive as merodiploids with $dnaA^+$ (Hansen et al. 1984); (*dnaA167* is not cold-sensitive in either circumstance). Informational suppression would be expected to result in a mixture of mutant and non-mutant proteins and thus mimic the situation in a heterozygote. There is however no evidence that *groE* can act as an informational suppressor. *λsdaA* does not suppress *dnaP*, *dnaB252*, or the two *dnaC* alleles tested, nor does pND5 suppress any of the nutritional markers of MM18 (data not shown).

There remain the possibilities that *groE* protein interacts directly with mutant *dnaA* protein molecules or that it serves to stabilize the initiation complexes within which they function. *GroE* was initially identified as essential for λ head morphogenesis, a process in which its role remains obscure. However, the fact that it is a very abundant cellular protein and that its active form has a complex 14 unit structure (Friedman et al. 1984) suggests that it may have a structural role. The initiation origin is known to bind many copies of the *dnaA* protein (Fuller et al. 1984). If the formation of a *dnaA* protein aggregate is important for initiation and if the suppressible Ts mutant proteins fail to form the required structure at restrictive temperatures, a protein capable of stabilising this structure could, when overproduced, suppress mutations defective in forming such an assemblage. Whether such an explanation is correct and why such large quantities of *groE* protein should be required awaits further work on the function of the *groE* proteins.

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