

Palindrome mediated inviability in Escherichia coli.

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To Leon.

**DECLARATION**

I hereby declare that I alone have composed this thesis and that, except where otherwise stated, the work presented within it is my own.

## ACKNOWLEDGEMENTS

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### ABSTRACT

In vivo deletion of a 3,200 base pair palindrome constructed in a lambda phage gave rise to phage carrying smaller palindromes of 500-700 base pairs (Leach and Stahl, 1983). In this work one of these smaller palindromes is characterised by sequencing and a mechanism proposed for the deletion event that led to its formation. This mechanism accounts for features of the sequence surrounding the endpoints of this deletion and palindrome deletions described by other authors.

A phage carrying this palindrome can plate on recBC sbcB sbcC cells but not on rec<sup>+</sup> and recA cells. This phage was used to study events in vivo in hosts where the phage is viable and inviable. The presence of the palindrome was shown to cause a decrease in the levels of actively replicating phage supercoils compared to the supercoils of a control palindrome free phage in cells where it was inviable. The decrease was shown not to be due to destruction of the phage DNA, but instead to slow replication of the palindrome containing DNA. This result distinguishes between the two major hypotheses proposed to account for palindrome mediated inviability.

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Appendix I In vivo loss of supercoiled DNA carrying  
palindromic sequence

ABBREVIATIONS

- AMPS - Ammonium persulphate  
Ap<sup>R</sup> - Ampicillin resistance  
BSA - Bovine serum albumin  
ddNTP - dideoxynucleoside triphosphate  
dNTP - deoxynucleoside triphosphate  
DTT - dithiothreitol  
EDTA - Diaminoethanetetra-acetic acid  
EtBr - Ethidium Bromide  
IPTG - Isopropyl  $\beta$ -D-thiogalactopyranoside  
kb - kilobase pairs  
m.o.i.- multiplicity of infection  
OD - optical density  
PEG - polyethylene glycol  
Pol I - polymerase I  
R.F. - replicative form  
SDS - sodium dodecyl sulphate  
SSB - single strand binding protein  
Tc<sup>R</sup> - Tetracycline resistance  
TEMED - N,N,N',N',- Tetramethylethylenediamine  
Tris - Tris (hydroxymethyl) aminomethane  
U - unit  
UV - ultra violet light  
w/v - weight to volume  
w/w - weight to weight  
Xgal - 5-Bromo-4-chloro-3-indoyl- $\beta$ -D-galactoside

## **Chapter 1**

### 1.1 Definition and introductory remarks

Palindromes are defined in a biological context as DNA sequences which possess two-fold rotational symmetry. A 'perfect' palindrome is symmetrical right up to the axis of symmetry, as distinct from inverted repeats which are separated by a non-symmetrical region. In the literature this distinction has become somewhat tenuous as many so called 'imperfect' palindromes have a non-symmetrical centre. The term palindrome is used in this work to describe a sequence with extensive two-fold symmetry, where any central asymmetry is small compared to the size of the repeats.

Interest in palindromes has arisen because of their potential for conformational flexibility, they are able to base pair intra-strand in addition to inter-strand. The discovery of such sequences in eukaryotes, together with sequences capable of forming Z-DNA, has led to a realization of the polymorphic nature of the DNA helix and a search for its biological significance. The contrasting discovery that long palindromes are absent from and confer inviability in prokaryotes has provoked interest for three major reasons. Firstly, because it provides a model system in which an observable effect (inviability) is mediated by a secondary structure. Secondly, the realization that palindromes might adopt a structure which is topologically equivalent to a recombination intermediate (the Holliday junction), together with the initial implication of certain recombination related nucleases in the fate of these

sequences aroused the hope that palindromes could be used to provide information on the process of recombination in prokaryotes. Thirdly the fact that many eukaryotic palindromes cannot be cloned into standard prokaryotic hosts for study, has stimulated a search for hosts in which inviability is overcome.

## 1.2 Distribution and nature of palindromes in eukaryotes and prokaryotes

Almost perfect palindromes of several hundred base pairs in length are found in the genome of eukaryotes, where they form several percent of the total DNA (Wilson and Thomas, 1974; Thomas et al., 1974). Their function is unknown and they have been shown to be randomly positioned with respect to unique and middle repetitive sequences in human DNA (Schmid and Deininger, 1975). Furthermore, studies in Xenopus have shown that unique DNA attached to isolated palindromic DNA can include all sequences and not just a small specific subfraction, suggesting that palindromes in Xenopus are inserted at different sites in the genome of different individuals (Perlman et al., 1976). This argues against a specific control function for many long palindromes. Long palindromes have also been found in the genomes of several eukaryotic viruses, including Simian virus 40, polyoma, herpes simplex I, pox and parvoviruses (Frisque, 1983; Stow and McMonagle, 1983; Weller et al., 1985; Baroudy et al., 1982; Tattersall and Ward, 1976; Hauswirth and Berns, 1979). In Simian virus 40, polyoma,

and herpes simplex I viruses, the palindromes are found at the origin of replication and are thought to act as the attachment sites for proteins involved in replication. Some palindromes in the eukaryotic genome may serve the same function, as it has been found that origin rich monkey DNA segments are enriched for inverted repeats (Zannis-Hadjopoulos et al., 1984). In pox and parvoviruses the palindromes are terminal and it has been suggested that they can fold back on themselves, providing a mechanism for filling in the gap remaining after excision of the RNA primer at the 5' end of the molecule during replication (Cavalier-Smith, 1974; Baroudy et al., 1982).

In prokaryotes, the best studied of which is E.coli, many short or imperfect regions of two-fold symmetry are found, several of which are involved in DNA-protein interactions, such as the replication origin of E.coli and several other prokaryotes (Hirota et al., 1979; Hobom et al., 1979; Zyskind et al., 1983), the lac operator, the CAP-protein binding site (Dykes, et al., 1975; Bertrand et al., 1975) and certain promoter regions (Walz and Pirrotta, 1975). In some cases it is the secondary structure of the messenger RNA transcribed from the symmetrical DNA which has important regulatory functions as occurs with the trp attenuator (Oxender et al., 1979) and the intercistronic regulatory elements found in many prokaryotic operons (Higgins et al., 1982). Perfect symmetrical regions of longer than 30 base pairs have not been found in the E.coli chromosome and long



palindromes are similarly absent from other naturally occurring E.coli replicons.

### 1.3 The behaviour of long palindromes in E.coli

The absence of palindromic molecules among multimers of fragments cloned in recombinant DNA experiments, provided an early indication that there were problems involved in introducing long palindromes into E.coli replicons (Collins and Hohn, 1978). Since then, numerous observations have been made of the difficulties in propagating long palindromes in E.coli, whether in vitro constructs (Sadler et al., 1978; Collins, 1981; Lilley, 1981; Collins et al., 1982; Hagan and Warren, 1982; Mizuuchi et al., 1982; Hagan and Warren, 1983; Leach and Stahl, 1983; Goodchild, et al., 1985; Müller and Turnage, 1986; Nakamura et al., 1986; Yoshimura et al., 1986), or naturally occurring eukaryotic palindromes (Perricaudet et al., 1977; Boisseay and Astell, 1985; Wyman et al., 1985). Few reports have been made on the effects of introducing palindromes into replicons of bacteria other than E.coli, however Behnke et al. (1979) have reported similar difficulties in propagating palindromes in Streptococcus, suggesting that the phenomenon is probably widespread in prokaryotes.

Those palindromes which are greater than approximately 300 base pairs in length show two features. Firstly, they confer inviability, so that their carrier replicon does not survive in E.coli and secondly, they are unstable and delete at varying frequencies (Collins,

1981; Collins et al., 1982; Hagan and Warren, 1983; Leach and Stahl, 1983; Nakamura et al., 1986; Yoshimura et al., 1986). Palindromes less than 300 base pairs in length are generally not lethal to their carrier replicon but are often unstable (Lilley, 1981; Courey and Wang, 1983; Müller and Turnage, 1986; Yoshimura et al., 1986). Inviability and instability are complex phenomena influenced by a large number of factors, such as length, sequence, the nature of the carrier replicon and the host genotype. However as inviability is a common property of all palindromes above a certain length and some degree of instability is associated with most, if not all palindromes, it seems probable that these phenomena are mediated by the palindromes ability to form a localized secondary structure. Before discussing inviability and instability in detail, the nature of this structure will be considered.

#### 1.4 Potential secondary structure of palindromic DNA sequences

In double stranded DNA a palindrome has the potential to form a structure with two stem and loop segments which will be bordered on either side by DNA with normal inter-strand base pairing. This conformation is termed a 'cruciform structure'. This structure will be thermodynamically unfavoured in linear or relaxed DNA as its formation involves the loss of several base pairing and stacking interactions in the loop and at the base of the stem. The cruciform conformation will however be

favoured in negatively supercoiled DNA, where the two DNA strands are underwound imposing a torsional stress on the molecule. Extrusion of the palindrome into a cruciform, results in the palindrome DNA being intra-wound in the looped out segment rather than inter-wound and this makes the more turns available for the rest of the molecule, thereby lowering the torsional stress. The thermodynamic favourability can be determined by subtracting the energy required to maintain the unpaired and unstacked base pairs at the loop and stem base from the energy released by the lowering of the torsional tension of the supercoiled molecule (Hsieh and Wang, 1975). It follows from this that the longer a palindrome and the higher the negative superhelical density, the more favourable the cruciform conformation is. Similarly any imperfections in the palindrome will lower the thermodynamic favourability of the conformation as they will result in energetically unfavourable structural perturbations.

### 1.5 Cruciform formation in vitro

There is a strong body of evidence to show that cruciform formation occurs in negatively supercoiled DNA in vitro, both with long palindromic constructs (Mizuuchi et al., 1981) and short naturally occurring prokaryotic palindromes (Lilley, 1980; Panayotatos and Wells, 1981). Mizuuchi et al., (1981) constructed a totally palindromic circular DNA molecule, by head to head ligation of the larger EcoRI-BamHI fragment of pBR322. Negative supercoiling by DNA gyrase resulted in some of the DNA

converting to a cruciform structure detectable by electron microscopy and gel electrophoresis. When the DNA was heated to 100°C, which initiates hairpin formation by destabilizing the helix and further supercoiled by DNA gyrase, up to 80% of the molecule could be extruded into hairpins. Lilley (1980) and Panayotatos and Wells (1981) demonstrated that the single strand specific endonuclease S1 introduced highly selective cleavages into supercoiled, covalently closed circular DNA molecules, such as ColE1, pBR322 and  $\phi$ X174 RF, but not into linear forms of these molecules. The ColE1 plasmid was cut uniquely by S1 nuclease and the cleavage site was found to be at the centre of an inverted repeat of 13 nucleotides separated by a non repetitious central 5 nucleotides. Supercoiled pBR322 and  $\phi$ X174 were also cleaved at specific sites and these sites were at the centre of inverted repeats. The authors concluded that the inverted repeats were forming cruciform structures in the supercoiled DNA, allowing the S1 to cleave the single stranded hairpin loops. The cruciform conformation of the inverted repeat of ColE1 is shown in figure 1.1. Similar techniques have been used to demonstrate cruciform transitions of several synthetic purine-pyrimidine such as d(AT)<sub>17</sub> (Haniford and Pulleybank, 1985) and d(CATG)<sub>10</sub> (Naylor et al., 1986). Other purine-pyrimidine sequences such as d(GC)<sub>n</sub> where n < 70 have been shown by a variety of techniques to preferentially adopt a Z conformation rather than a cruciform conformation in negatively supercoiled DNA and this may be the favoured conformation

9

**Figure 1.1**

DNA sequence of ColEI around the site cleaved by S1, drawn in a cruciform structure (sequence taken from Lilley, 1980 and Oka et al.,1979). The single stranded loops are the target sites for the enzyme.

A  
 A T  
 A G  
 C-G  
 C-G  
 T-A  
 A-T  
 A-T  
 C-G  
 G-C  
 A-T  
 T-A  
 C-G  
 C-G  
 T-A  
 G-C  
 5'...ATTGATATAAAAA CAAACAAAGTAGAT...3'  
 3'...TAACTATATTTTT GTTTGTTTCATCTA...5'  
 C-G  
 A-T  
 G-C  
 G-C  
 A-T  
 T-A  
 C-G  
 G-C  
 T-A  
 T-A  
 A-T  
 G-C  
 G-C  
 T C  
 T A  
 T

for several eukaryotic palindromes (Nordheim et al., 1982; Peck et al., 1982; Singleton et al., 1982; Stirvidant et al., 1982; Cantor and Efstratiadis, 1984; Frank-Kamenetskii and Vologodskii, 1984).

### 1.6 Cruciform formation in vivo

Although cruciforms have been demonstrated in vitro there has been little evidence to indicate that they exist in vivo. Experiments in vitro have been performed on extracted DNA which has a superhelical density of  $-0.05$ , whether from prokaryotic or eukaryotic sources. However in vivo eukaryotic DNA is wound around nucleosomes and is under no net torsional stress, although local releases of torsional tension may occur transiently, as in the structural transition from inactive to transcriptionally active chromatin (Luchnik et al., 1982). This release of torsional stress could lead to the conformational transition of palindromes into cruciforms.

In prokaryotes, the DNA is maintained in a negatively supercoiled state by the action of DNA gyrase (Gellert et al., 1978). Until recently the superhelical density of prokaryotic DNA in vivo was thought to be the same as that in vitro, that is  $-0.05$ . Recent evidence (reviewed in Lilley, 1986) suggests that the superhelix density inside the bacterium is considerably lower and probably about  $-0.025$ . Prokaryotic DNA appears to be complexed with HU proteins in a structure similar to that of eukaryotic chromatin, but unlike eukaryotic chromatin

the HU complex fails to relax all of the torsional stress, retaining a level of supercoiling about half that of the protein free DNA (Broyles and Pettijohn, 1986). This revised estimation of in vivo superhelical density means that it is improbable, given the results of statistical mechanical calculations (Vologodskii and Frank-Kamenetskii, 1982), that those naturally occurring prokaryotic palindromes, such as those found in pBR322 which have been shown to cruciform in vitro would do so in vivo. Many longer palindromes can however form thermodynamically stable cruciforms at this superhelical density and the main argument against their formation in vivo is that advanced by Courey and Wang (1983) and Gellert et al., (1983). These authors concluded that because cruciform formation in vitro is very slow even at high negative superhelicities, unless the DNA is subjected to conditions that destabilize base pairing, it is therefore unlikely to occur under physiological conditions. This argument does not preclude the catalysis of cruciform formation in vivo by strand separation brought about by proteins. Indeed in vivo effects mediated by palindromes, such as inviability and instability, have been shown to be independent of absolute central symmetry, as insertion of a central asymmetry of up to 50 base pairs does not reverse these effects (Warren and Green, 1985). This makes it very improbable that these phenomena are due to uncatalysed cruciform extrusion.

Attempts to detect cruciform formation in vivo



with two small palindromes (68 and 66 base pairs) resulted in the conclusion that these palindromes did not form a cruciform in vivo (Courey and Wang, 1983; Sinden et al., 1983), however the recent revised estimation of superhelical density of prokaryotic DNA in vivo makes it uncertain whether the cruciform conformation of these palindromes would be favoured. Similarly much of the evidence supporting the existence of cruciforms in vivo has been plagued with ambiguities, as many experimenters have not taken precautions to avoid triggering cruciform extrusion during DNA extraction.

Cruciform formation in vivo has been shown clearly with one specialised case with a palindrome consisting of the simple repeating dinucleotide d(AT)<sub>34</sub>. This palindrome does not cruciform inside the bacterium under normal conditions (Greaves et al., 1985), but when protein synthesis is blocked by chloramphenicol, the reduced availability of DNA binding proteins appears to cause the in vivo superhelical density to rise, resulting in transition to the cruciform (Haniford and Pulleybank, 1985). Studies in vitro on d(AT) sequences have shown that they have an abnormal underwound conformation in linear DNA molecules and this altered conformation is subject to easy torsional deformation, so that such sequences do not have the usual kinetic barrier to cruciform transition (McClellan et al., 1986). Sequences of d(AT)<sub>n</sub> where n>37 can form cruciforms at superhelical densities of -0.025 and as there is no kinetic barrier to the transition it seems probable that

they do so in vivo. A sequence of d(AT)<sub>37</sub> has been shown to be unstable in an E.coli replicon (R. Patient cited in Lilley, 1986). As previously mentioned these sequences are special cases as the palindrome lacks a kinetic barrier to cruciform formation, so in vivo studies on such sequences may not be applicable to other palindromes. In general it appears that if the majority of palindromes do form cruciforms in vivo, this transition would need to be catalysed.

If most palindromes do not form a cruciform in vivo, then it is possible that they mediate their effects by forming hairpins in single stranded regions of DNA, such as occur due to the opening of paired strands during replication. There is no kinetic barrier to the formation of single strand hairpins and they are thermodynamically favoured due to their large free energies of formation (Tinoco et al., 1973). E.coli single strand binding protein may prevent the transition, but the instability of small palindromes which would be unlikely to cruciform, makes it probable that at least some biological effects of palindromes are mediated by single strand snapbacks.

### 1.7 Factors affecting palindrome instability

Of the two major biological consequences of palindromes, inviability and instability, instability is perhaps the better studied and understood. Suggestions that even short imperfect palindromes have intrinsic instability were made because of the association of

naturally occurring palindromes with spontaneous deletions (Albertini et al., 1982; Glickman and Ripley, 1984; Schaaper et al., 1986). Studies with long palindromic constructs have shown that palindromic deletion is recA independent and can be exact or partial (Collins, 1981; Collins et al., 1982). Partial deletion usually occurs equally or almost equally about the axis of symmetry, so that the remnant is still palindromic, either totally or more frequently containing a short asymmetric centre (Collins, 1981; Collins et al., 1982; Leach and Stahl, 1983; Yoshimura et al., 1986). This asymmetric centre may contribute to the stability of the palindromic remnant. Warren and Green (1985) showed that stability could be restored to a large (>1kb) unstable palindrome if an asymmetric centre of 150 base pairs was inserted between the repeats.

Deletion usually occurs between direct repeats, which may be as short as 3 base pairs, within the palindrome or bordering it (Collins et al., 1982; Yoshimura et al., 1986; DasGupta et al., 1987). An interesting observation is that when deletion occurs between short direct repeats within the palindrome, one or both of these repeats are sometimes themselves in the centre of a short region of two-fold symmetry i.e. a 'mini-palindrome' within the larger palindrome (Collins et al., 1982; Boissy and Astell, 1985; Yoshimura et al., 1986; also see chapter 3 for a possible explanation of this observation). This observation has also been made in analysing 'nearly precise' excision events of the

transposon Tn10 (Foster et al.,1981).

Deletion appears to occur more frequently in replicons with a single strand intermediate in their life cycle, perhaps reflecting the increased opportunity for single strand hairpin formation (Müller and Turnage, 1986; Leach et al.,1987).

Instability is strongly influenced by DNA sequence, not only of the palindrome itself, but also of the surrounding DNA. Hagan and Warren (1983) observed that the same palindrome formed in different carrier plasmids showed considerable differences in deletion frequency. The importance of local sequence in palindrome deletion was investigated in detail by DasGupta et al.(1987), who constructed pBR322 plasmids carrying palindromes of 22, 32 and 90 base pairs in length, flanked by 9 base pair direct repeats, inserted at different sites in the ampicillin resistance gene. Deletion of the palindrome and one direct repeat resulted in reversion to ampicillin resistance. Deletion frequencies of the 22 base pair palindrome ranged from  $10^{-8}$  to  $10^{-6}$  depending on where in the gene the palindrome was inserted. Elongation of this palindrome from 22 to 90 base pairs stimulated this deletion by factors ranging from 8 to 18,000 fold among the sites tested. A markedly different deletion frequency of the 90 base pair palindrome was observed between insertion sites separated by only one base pair, the deletion frequency between the neighbouring sites differed by a factor of

10<sup>4</sup>. This demonstrates the considerable effect of subtle changes in local DNA sequence on palindrome stability.

### 1.8 Hypotheses to account for palindrome instability

There have been two major hypotheses put forward to account for the instability of palindromes and the involvement of short direct repeats in their deletion. Both models showing the complete excision of a palindromic DNA sequence bordered by direct repeats are outlined in figure 1.2. The first model, suggested by DasGupta et al., (1987) shows the extrusion of the palindrome into a cruciform structure, which is cleaved by a conformation specific nuclease. This is followed by limited 3' to 5' exonucleolytic degradation of the DNA and annealing of the complementary sequences from the first and second direct repeats. Repair synthesis completes the process and exact deletion of the palindrome is accomplished.

The second model suggested by Collins (1981) and DasGupta et al. (1987) is an extension of the 'strand slippage' mechanism postulated by Streisinger et al. (1966) originally invoked to explain frameshift mutations. In this model single strand hairpins are formed in the replication fork, which impede copying of the template strand. The nascent DNA strand becomes separated from the first copy of the direct repeat and reassociated with the second copy. Synthesis then resumes. The hairpin and single strand region may then be removed by cleavage and degradation or merely lost

**Figure 1,2**

Models of deletion formation (adapted from DasGupta et al.,1987)

(i) (a) The palindrome is extruded into a cruciform structure in supercoiled DNA. Direct repeats are indicated by arrows. A conformation specific nuclease cleaves the base of the hairpins.

(b) Region with the hairpins removed.

(c) Limited resection by a 3' to 5' exonuclease, followed by annealing of complementary sequences from each direct repeat.

(d) Repair synthesis to produce deletant which has lost the palindrome and one copy of the direct repeat.

(ii) (a) The palindrome forms a hairpin in a single stranded region of the replication fork. Direct repeats are indicated by arrows.

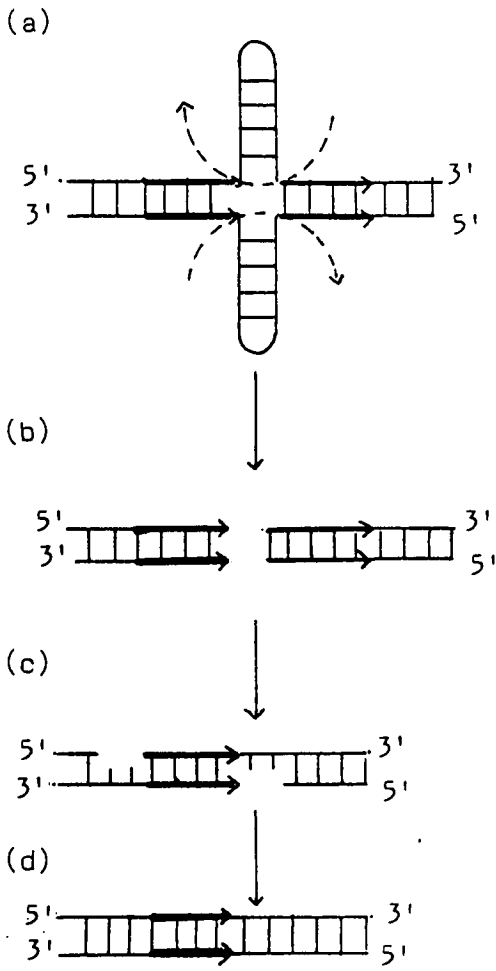
(b) The hairpin impedes copying of the template strand by DNA polymerase III.

(c) The end of the nascent DNA strand becomes separated from the first copy of the direct repeat and reassociates with the second copy. The first direct repeat is now an unpaired (single strand) region.

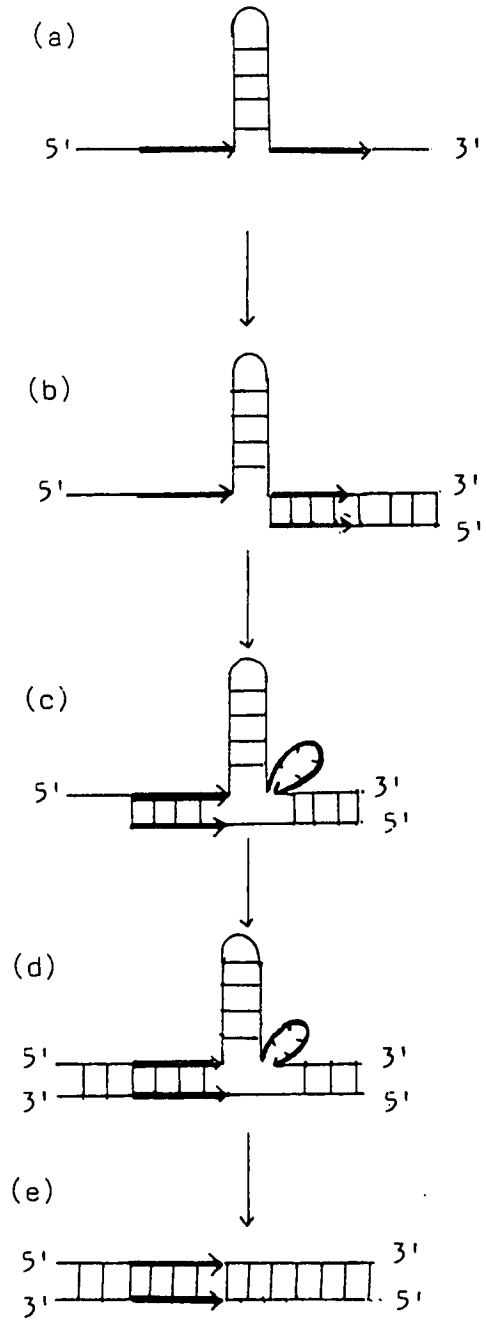
(d) Synthesis resumes.

(e) The hairpin and single strand region are either removed by cleavage or lost following another round of replication to give a deletant which has lost the palindrome and one copy of the direct repeat.

(i)



(ii)



following another round of replication. This model is more attractive than the first model, because it does not depend upon cruciform formation and many palindromes which would be unlikely to cruciform in vivo are unstable. Also this model accounts for the observation of Shurvinton et al.(1987), who found that following infection of a rec<sup>+</sup> host by a lambda phage carrying a large, 8,400 base pair palindrome, the palindrome could be recovered intact in phage particles carrying unreplicated chromosomes, but not from those with replicated chromosomes (an alternative explanation however, is that replication drives the extrusion of the palindrome which is then attacked according to the first hypothesis).

The 'strand slippage' model has been previously invoked to explain the 'normal' recA independent generation of deletions between direct repeats in the absence of palindromes (Farabaugh et al.,1978). Palindromes could increase the frequency of 'normal' deletion between direct repeats in two ways. Firstly the slowing down of replication due to a hairpin impediment could facilitate separation of the end of the nascent DNA strand from its template and secondly, the hairpin conformation could bring the two repeats closer together spatially, increasing the probability of the nascent DNA strand encountering and annealing to the second direct repeat.



### 1.9 Factors affecting palindrome mediated inviability

As previously mentioned, palindrome mediated inviability is length dependent and usually a palindrome must be greater than 300 base pairs in length to be lethal to its carrier replicon. Two smaller palindromes of 146 and 147 base pairs, while not causing inviability, have however been shown to cause a reduced copy number of their carrier plasmids (Warren and Green, 1985), suggesting that inviability is not an 'all or nothing' phenomenon.

The more imperfect a palindrome is and the longer any asymmetric region between its repeats is, the less likely it is to confer inviability. Warren and Green (1985) restored viability to a plasmid carrying a palindrome of over 1kb in length, by inserting 50 base pairs of non symmetrical DNA in the centre of the palindrome.

Host genotype also has a significant effect on palindrome conferred inviability. Leach and Stahl (1983) constructed a lambda phage with the non essential EcoRI B fragment replaced by a 3,200 base pair palindrome, consisting of an inverted duplication of the fragment from the unique XbaI site to the second EcoRI site of phage lambda. This phage was found to plate with the same efficiency as a palindrome free control phage on strains carrying recB, recC, sbcB and sbcC mutations. On rec<sup>+</sup> strains the palindrome plated with a much reduced frequency (<10<sup>-2</sup>). The 3,200 base pair palindrome was unstable in the recBC sbcB sbcC strain, as up to 20% of

the progeny phage obtained from plaques on this strain were able to plate on a rec<sup>+</sup> strain, suggesting that they had deleted the palindrome. Physical analysis of the DNA of phages grown as plate stocks on a recBC sbcB sbcC strain showed that the restriction fragment corresponding to the perfect palindrome was absent, apparently from the entire population of phage particles, although a large proportion were still unable to plate on rec<sup>+</sup> strains. The phage which were unable to plate on rec<sup>+</sup> hosts had retained a palindromic structure of about 600 base pairs, as seen by the presence of snapbacks in single strands of the DNA of these phages, when viewed under the electron microscope. This shorter palindrome was much more stable in recBC sbcB sbcC hosts, deleting at a frequency of about  $10^{-4}$  (Leach *et al.*, 1987) and high titre stocks of phage containing it could be grown on these hosts.

A similar observation was made with a palindrome of approximately 8kb cloned in bacteriophage lambda (Shurvinton *et al.*, 1987), this phage could grow on recBC sbcB sbcC hosts, but was unstable and deleted, either completely or leaving smaller much more stable palindromes of approximately 700 base pairs in length. The original phage containing an 8kb palindrome formed only tiny plaques on the recBC sbcB sbcC host, but the phages containing the 700 base pair deleted palindrome formed much larger plaques, suggesting that viability in recBC sbcB sbcC backgrounds is also influenced by palindrome length. The palindromic remnants described by Leach and Stahl and Shurvinton *et al.* are very similar in

size. This may be the size at which the palindrome containing phage grows as well as its palindrome free counterpart in recBC sbcB sbcC hosts, so that any palindrome free phages generated by deletion do not overgrow the palindrome phage. The fact that the palindrome remnants are all of similar size may also reflect some aspect of the deletion process.

Other observations have been made on the increased viability of palindrome carrying lambda phages in recBC sbcB sbcC hosts. Wyman *et al.* (1985) reported that approximately 9% of the phages from a human genomic library made in lambda vectors failed to grow on rec<sup>+</sup> hosts but grew on recBC sbcB sbcC hosts. When DNA from four such phages was examined under the electron microscope, each was shown to have at least a few snapback structures consistent with perfect or nearly perfect palindromes 200-500 base pairs in length. The control clones which grew on rec<sup>+</sup> hosts did not show evidence of secondary structures in the human DNA.

Two observations have been made relating to the effect of recBC sbcB sbcC mutations on plasmids carrying palindromes. Collins *et al.* (1982) observed that recBC sbcB sbcC hosts apparently reduced the frequency of precise excision of palindromes bounded by short direct repeats and Boissey and Astell (1985) showed that a plasmid carrying a 206 base pair imperfect palindrome, unstable in wild type hosts could be stably propagated in a recBC sbcB sbcC recF strain. While it is tempting to assume that these two observations show that the recBC

sbcB sbcC genotype affects instability as well as inviability, it is possible that the presence of these mutations in these two cases merely increases the viability of the palindrome carrying replicon relative to its non palindromic deletion derivative.

Before discussing recBC, sbcB and sbcC further and considering the effect of other recombination genotypes on palindrome mediated inviability it is worthwhile briefly reviewing the recombination pathways of E.coli.

#### 1.10 Recombination pathways in E.coli

In E.coli recombination can proceed by several pathways. All of these pathways rely on the product of the recA gene, therefore recA mutants exhibit very low levels of recombination, about  $10^{-4}$  to  $10^{-6}$  of the wild type frequency (Clark and Margulies, 1965). The RecA protein is a DNA dependent ATPase that promotes homologous pairing of duplex DNA molecules with single stranded or partly single stranded molecules (Weinstock et al., 1979; Shibata et al., 1979). E.coli single strand binding protein and topoisomerase I are also involved in this reaction, which is reviewed by Radding (1985). Strand transfer by the RecA protein produces a 'Holliday' junction (DasGupta et al., 1981) a structure first proposed by Robin Holliday as a molecular intermediate in genetic recombination (Holliday, 1964). The recA mutation also affects repair, mutagenesis and cell division, as the RecA protein in its capacity as a specific DNA-

dependent protease, plays a major role in the SOS response (Little and Mount, 1982).

The major pathway of recombination in E.coli requires the products of the recA, recB, recC and recD genes. Mutations in either the recB or the recC gene result in reduced recombination proficiency (about  $10^{-2}$  of the wild type frequency), reduced ability to recover from DNA damage, reduced capacity to degrade foreign and damaged DNA and segregation of non viable progeny (Clark, 1973; Simmon and Lederberg, 1972; Capaldo Kimball and Barbour, 1971). The existence of recD was discovered relatively recently as a class of mutants which were recombination proficient but were sensitive to phage T4 gene 2 mutants (Chaudhury and Smith, 1984). This suggested that these mutants, unlike wild type cells, were unable to degrade the injected DNA of the T4 gene 2 mutants. The recD mutation was found to form an operon with recB (Amundsen et al.,1986; Finch et al.,1986).

The recB, recC and recD genes code for proteins of 135kDa, 125kDa and 67kDa respectively (Hickson and Emmerson, 1981; Umeno et al.,1983; Dykstra et al.,1984; Finch et al.,1986). Together these products make up the RecBCD enzyme, also known as Exonuclease V (Tomizawa and Ogawa, 1972). This enzyme exhibits several different activities in vitro, it is an ATP dependent double stranded exonuclease (Oishi, 1969; Goldmark and Linn,1972), a single stranded DNA exo and endonuclease (Goldmark and Linn, 1970) and also an ATP dependent DNA unwinding enzyme (Rosamond et al.,1979; Taylor and Smith,

1980). The enzyme of recD mutants has been shown to lack all detectable nuclease activity, both in vivo and in vitro (Chaudhury and Smith, 1984).

The unwinding reaction of the RecBCD enzyme has been studied in vitro (Taylor and Smith, 1980), where it appears that the RecBCD enzyme attaches to one end of a molecule and assimilates the DNA, subsequently releasing it at a slower rate, creating single strand loops which grow in size as the enzyme travels along the molecule. These single strand loops are potential substrates for the RecA homologous pairing reaction.

The RecBCD recombination pathway is stimulated by the presence of Chi sites (for a review see Smith, 1985). These are sites which have the sequence 5'GCTGGTGG 3' (Smith et al., 1981a; Triman et al., 1982) and are cleaved by purified RecBCD enzyme in vitro (Ponticelli et al., 1985). Cleavage of these sequences in vivo, coupled to unwinding by the RecBCD enzyme may promote strand transfer of DNA by the RecA protein due to the provision of a single stranded tail (Smith et al., 1981b). Chi sites have no detectable activity in recD mutants (Chaudhury and Smith, 1984).

Mutations in recB or recC can be suppressed by sbcA mutations, which turn on the recE gene of a cryptic prophage (rac), present in some strains (Barbour et al., 1970; Fouts et al., 1983; Willis et al., 1983). This results in a nuclease called Exonuclease VIII being made, which closely resembles the nuclease coded for by the lambda red genes (Kushner et al., 1974; Joseph et

al.,1983).

Mutations in recB or recC can also be suppressed by sbcB mutations. These mutations result in the inactivation of a nuclease known as Exonuclease I (Kushner et al.,1971). This exonuclease has been purified and shown to be a 140kDa dimer with a 3' to 5' exonuclease activity and a high specificity for single stranded as oppose to double stranded DNA (Lehman and Nussbaum, 1964; Ray et al., 1974). Its precise function is unclear, but its inactivation in recBC sbcB cells results in the recF pathway of recombination being fully operational. The recF pathway requires the products of the recA, recF, recJ, recN, recQ and ruv genes (Horii and Clark, 1983; Lovett and Clark, 1983; Lloyd et al.,1984; Nakayama et al.,1984) and is subject to lexA regulation (Armengod,1982; Lloyd and Thomas, 1983; Lovett and Clark, 1983). It has been suggested that Exonuclease I may attack a recombination intermediate in the recF pathway, which is why its absence leads to recombination proficiency.

Recently it was discovered that commonly used recBC sbcB strains also carried an additional mutation in a new gene designated sbcC (Lloyd and Buckman, 1985). The sbcC mutation on its own had no effect on the phenotype of rec<sup>+</sup>, recB or recC strains, which indicated that it is the sbcB mutation which was critical for the restoration of recombination proficiency. However the presence of sbcC in recBC sbcB cells is essential to fully suppress the mitomycin C sensitivity, recombination deficiency and

segregation of non viable progeny associated with the recBC genotype. In the absence of sbcC, recBC sbcB cultures grow very slowly and contain many inviable cells, which leads to the rapid accumulation of fast growing recBC sbcB sbcC mutants. The product of the sbcC gene has not been identified.

### 1.11 The effect of genotype on the plating of palindrome containing lambda phage

As previously described, lambda phage containing palindromes of 3,200 and 600 base pairs (Leach and Stahl, 1983), 8,000 and 700 base pairs (Shurvinton et al.,1987) and 200-500 base pairs (Wyman et al.,1985) can plate on recBC sbcB sbcC hosts but not on rec<sup>+</sup> hosts. The recBC sbcB sbcC strains used by these authors were also RecD<sup>-</sup> as they possessed the recB21 mutation which is polar on recD. The effect of these mutations separately has also been examined (Leach and Stahl, 1983; Wyman et al.,1985; Leach et al.,1987; D. Leach and A. Chalker, personal communications).

Leach and Stahl (1983) showed that the 3,200 base pair palindrome containing phage would not plate on hosts containing mutations in only recBC. In contrast the 600 base pair palindrome phage plated with almost normal efficiency (compared to its isogenic palindrome free control) on recBC and recD hosts (Leach et al.,1987; D. Leach, personal communication). The plaques on these strains were however very small and contained a high proportion of phage which had deleted the palindrome.



The product of the lambda gam gene inhibits the nuclease activity of the RecBCD enzyme (Greenstein and Skalka, 1975; Freidman and Hayes, 1986). The palindrome containing phages so far described have all been gam<sup>-</sup>. Leach et al (1987) have shown that the suppression of the gam210 mutation of the 600 base pair palindrome containing phage allows it to plate on rec<sup>+</sup> hosts.

The absence of the RecBCD nuclease activity in these three cases ( recBC<sup>-</sup>, recD<sup>-</sup> and gam<sup>+</sup> ) probably affects the plating ability of the phages non specifically and does not represent a genuine alleviation of the inviability caused by the presence of the palindrome. Under conditions where the RecBCD nuclease activity is absent, phage lambda can replicate by 'rolling circle' in addition to theta replication, which results in more packagable products and a larger burst size (see Smith, 1983 for a review of the alternative modes of lambda replication and also chapter 4 of this work). This non specific increase in the levels of the 600 base pair palindrome containing phage may permit it to undergo a few rounds of growth and cell lysis, generating faster growing deletion derivatives in the process and permitting a small plaque to be formed.

The presence of the sbcB mutation alone, did not allow plating of the 3,200 base pair palindrome or the 600 base pair palindrome containing phages (Leach and Stahl, 1983; Leach et al.,1987), but Wyman et al. (1985) reported a slightly beneficial effect of the sbcB mutation on the plating ability of some of their

palindrome containing phages and the plaque size is increased slightly on addition of sbcB to a recD host (D. Leach, personal communication. This suggests that sbcB may play a minor role in the plating of these phages, although whether it is a specific interaction with the palindrome is unclear.

Leach and Stahl (1983) demonstrated that the 3,200 base pair palindrome containing phage did not plate on a recBC sbcA host and that the presence of the lambda red genes did not affect its plating ability on rec<sup>+</sup> or recBC sbcB sbcC hosts, suggesting that the recE or lambda red recombination pathways probably play no part in determining viability of the phage.

In recBC sbcB sbcC cells the recF pathway of recombination is operational. However additional mutations in recF in recBC sbcB sbcC cells were shown not to affect the plating ability of the 3,200 base pair palindrome containing phage (Leach and Stahl, 1983) and similarly additional mutations in recA, recF, recJ, recN, ruv and lexA were found not to affect the plating of the 600 base pair palindrome containing phage on recBC sbcB sbcC cells (Leach et al., 1987). These results suggest that it is not the activation of the recF pathway or some component of it that permits the palindrome containing phage to plate.

Until very recently no work had been done on the effect of the sbcC mutation alone on the plating of palindrome containing phages. Leach and Stahl (1983) were unaware of its existence at that time and it has been

neglected by subsequent authors who have concentrated on why the absence of the recBC and sbcB gene products is important. A. Chalker (personal communication) has however shown that the 600 base pair palindrome containing phage will plate on hosts mutant only in sbcC and phages obtained from plaques formed on these hosts still retain the palindrome. From this result it appears that the recBC and sbcB mutations may be incidental and it is the sbcC mutation which is really important in determining viability of the palindrome containing phages.

#### 1.12 Hypotheses to account for palindrome mediated inviability

Several hypotheses have been put forward to explain the inviability of palindrome carrying replicons. One of the earliest explanations of palindrome mediated inviability (Lilley, 1981) was that the loss of the free energy of supercoiling into cruciform structures, results in such energy being unavailable for driving process requiring strand separation, such as transcription (Wang, 1974; Richardson, 1974). However, the native DNA superhelical density is now known to be very tightly regulated in vivo by the activities of DNA gyrase and topoisomerase I (Gellert et al., 1976; Sugino et al., 1978; Menzel and Gellert, 1984; Goldstein and Drlica, 1984; Richardson et al., 1984). Any local transition which results in a reduction of the net number of negative supercoils without affecting the linking number

of the molecule (such as would occur if cruciform extrusion took place), results in the regulatory system reducing the linking number of the molecule so that the level of supercoiling preceding the transition is re-established.

The two currently most favoured hypotheses are that either the presence of the palindrome results in cleavage of its carrier replicon, due to mistaken recognition of the cruciform for a Holliday junction (Mizuuchi et al., 1982; Leach and Stahl, 1983) or that the palindrome forms a secondary structure which interferes with replication (Bolivar et al., 1977; Hagan and Warren, 1983; Leach and Lindsey, 1986). These two hypotheses will be discussed further.

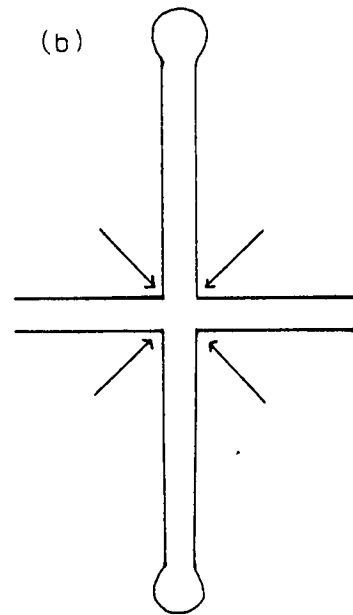
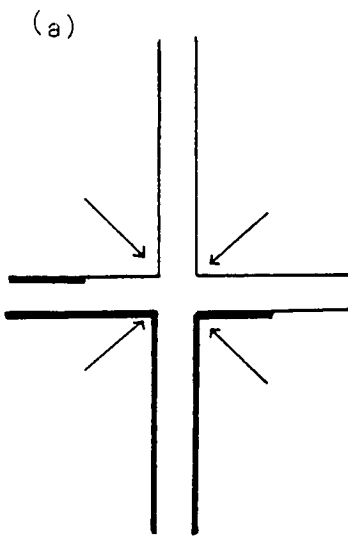
The base of a cruciform structure is topologically indistinguishable from a Holliday junction (Mizuuchi et al., 1982; Lilley and Kemper, 1984), a central intermediate in recA mediated recombination, mentioned earlier. The two structures are presented for comparison in figure 1.3. Holliday junctions are resolved during recombination by cleavage across the base to generate two recombinant molecules. The T4 endonuclease VII has been shown to resolve Holliday junctions in vivo (Frankel et al., 1971; Kemper and Brown, 1976; Kemper and Janz, 1976; Nishimoto et al., 1979) and to cleave cruciforms across the base in vitro (Lilley and Kemper, 1984). Similar properties have been shown for the T7 gene 3 product (deMassy et al., 1984) and an enzyme from Saccharomyces cerevisiae (West and Körner, 1985). It has

**Figure 1.3**

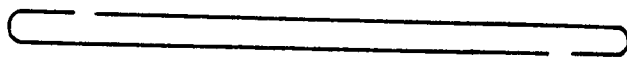
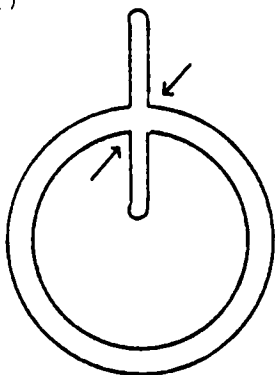
(i) (a) The Holliday structure drawn in the trans configuration and (b) the cruciform conformation presented for comparison. As illustrated cleavage can occur across the junction diagonally in either of two ways to resolve the Holliday junction or destroy the palindrome carrying replicon.

(ii) Cleavage and linearisation of the palindrome containing replicon.

(i)



(ii)



not been determined which enzyme is responsible for resolving Holliday junctions in E.coli. Leach and Stahl (1983) postulated that the products of the recBCD and sbcB genes resolve Holliday junctions and hence cleave cruciforms in E.coli, explaining the viability of the 3,200 and 600 base pair palindrome containing phages in recBC sbcB sbcC hosts. Now that sbcC is thought to be the significant mutation, it is still possible to postulate that the sbcC gene product has this ability.

The second hypothesis postulates that the palindrome impedes replication by the formation of a hairpin in the replication fork. DNA synthesis in E.coli is carried out mainly by DNA polymerase III, a complex enzyme consisting of at least seven subunits (McHenry and Kornberg, 1977; McHenry and Crow, 1979; McHenry, 1982). Site specific pausing of DNA polymerase III at regions capable of forming hairpins, has been detected in vitro (LaDuca et al.,1983). E.coli single strand binding protein (SSB) was shown to overcome this pausing by removing these hairpin structures (LaDuca et al.,1983). While SSB would be expected to remove hairpins in vivo it is possible that it cannot do so immediately, perhaps because of exhaustion of the available supply, as there are only an estimated 300-500 copies of the SSB tetramer per cell (Weiner et al.,1975; Cuozzo and Silverman, 1985).

Interference of a palindrome with replication in vivo has been shown in one instance. Bolivar et al.,(1977a) found that replicative intermediates of a

small plasmid, containing two lac operator fragments arranged as a palindrome, formed in the presence of chloramphenicol. These intermediates appeared as 'eye' like structures under the electron microscope. Sequencing these replicative structures they found that they started at the origin of replication and appeared to switch strands within a ten base pair region and replicate back in the opposite direction, terminating near the origin. The region at which they switched strands was at the centre of the two operator segments, leading the researchers to conjecture that a hairpin structure had formed in this region disrupting DNA synthesis and causing DNA polymerase to replicate back along the opposite strand. This strand switching occurred only in the presence of chloramphenicol, which can be explained in at least three ways. Firstly, plasmid synthesis in the presence of chloramphenicol may be carried out by DNA polymerase I rather than DNA polymerase III and it may be that the palindromes secondary structure interferes with replication by the former but not the latter polymerase, this is the explanation proffered by the authors. Secondly, as chloramphenicol results in a rise in the in vivo superhelical density due to the reduced availability of DNA binding proteins as previously described, this may result in the palindrome forming a cruciform which then impedes replication. Thirdly the prevention of protein synthesis by chloramphenicol may result in a reduced level of the proteins needed to destabilize hairpins.



Both the 'cruciform cleavage' and the 'replication interference' hypotheses will successfully account for palindrome mediated inviability. The first hypothesis requires that cruciform extrusion occurs in vivo and predicts that the palindrome carrying replicon is destroyed. The second hypothesis requires that hairpins are present in the replication fork, which impede replication, but do not necessarily result in the destruction of the palindrome carrying DNA.

## Chapter 2

## 2.1 Materials

### 2.1.1 Bacteria, phages and plasmids

The bacterial strains used in this work are described in table 2.1. For strains which were used solely to prepare phage stocks only the relevant genotype is given, the full genotype of all other strains is listed.

The bacteriophages and plasmids used in this work are described in tables 2.2 and 2.3 respectively.

### 2.1.2 Bacteriological media

The following quantities are for 1 litre volumes, made up in distilled water and sterilized by autoclaving at 15lb/in<sup>2</sup> for 20 minutes.

L broth Difco Bacto Tryptone, 10g; Difco Bacto Yeast Extract, 5g; NaCl, 10g. Adjusted to pH7.2 with NaOH.

L broth p/c L broth supplemented with 0.2% maltose and 5mM MgSO<sub>4</sub>.

L agar As for L broth, but with the addition of 15g of Difco Agar per litre.

BBL agar Trypticase (Baltimore Biological Laboratories), 10g; NaCl, 10g; Difco Agar, 10g.

**Table 2.1** Bacterial strains used in this work

## Notes

1. These strains are derivatives of AB1157 genotype F<sup>-</sup> DEL(gpt-pro)62 argE3 his-4 leu-6 thr-1 ara-14 galK2 lacY1 mtl-1 thi-1 supE44 rpsL31 tsx33
2. These strains are derivatives of W3101, genotype F<sup>-</sup> galT1
3. JL32 was constructed by conjugation of Hfr strain GM81 with JC9387 (GM81 is described fully in McGraw and Marinus, 1980).
4. These strains were used to prepare lambda phage stocks.
5. These strains were used in lambda supercoil extraction experiments.
6. These strains were used in cloning experiments.

Strain	Notes	Description	Source
JC9387	1,5	<u>recB21</u> <u>recC22</u> <u>sbcB15</u> <u>su<sup>-</sup></u> <u>sbcC</u> <u>RecD<sup>-</sup></u>	F. Stahl
JC9937	1,5	<u>rec<sup>+</sup></u> <u>su<sup>-</sup></u>	F. Stahl
DL187	1,5	JC9937 lysogenic for $\lambda$ JMC082	D. Leach
DL157 <sub>5</sub>	1	JC9387 Hfl <sup>-</sup>	D. Leach
DL190	1,4	DL157 <sub>5</sub> lysogenic for $\lambda$ JMC249	D. Leach
DL262	1,4	DL157 <sub>5</sub> lysogenic for $\lambda$ DRL110	D. Leach
JL12	1,4	DL262/pMB1	This work
JC8679	1	<u>recB21</u> <u>recC22</u> <u>sbcA20</u> <u>supE44</u> <u>RecD<sup>-</sup></u>	F. Stahl
JL27	1	JC8679/F <sup>+</sup> (Tra <sup>+</sup> <u>proA<sup>-</sup></u> <u>proB<sup>+</sup></u> <u>lacI<sup>Q</sup></u> <u>lacZ-del-M15</u> )	This work
JL28	1,4	JL27/pTP166	This work
JL32	1,3,5	JC9387 <u>dam3</u> Xyl <sup>+</sup> Str <sup>S</sup>	This work
GM119		<u>dam3</u> <u>dcm6</u> <u>metB1</u> <u>galK2</u> <u>galT22</u> <u>lac</u> <u>Y1</u> <u>tsx78</u> <u>thr1</u> <u>tonA31</u> <u>mtl1</u> <u>supE44</u>	M. Marinus
JL30	5	GM119 <u>su<sup>-</sup></u>	This work
JL33	5	JL30 lysogenic for $\lambda$ wild type	This work
W3101 RecA <sup>-</sup>	2,5	<u>recA13</u> <u>su<sup>-</sup></u>	N. Murray
JL4	2,5	W3101 RecA <sup>-</sup> /pBR322	This work
JL9	2,5	JL4 lysogenic for $\lambda$ wild type	This work
JM1	4	<u>recB21</u> <u>sbcA20</u> <u>supF</u> <u>RecD<sup>-</sup></u>	F. Stahl
JL5	4	JM1/pMB1	This work
JM83	6	<u>ara</u> DEL( <u>lac-pro</u> ) <u>rpsL</u> <u>thi</u> <u>ø80</u> <u>lacZ-del-M15</u> <u>rec<sup>+</sup></u>	D. Finnegan
JM101	6	DEL( <u>lac-pro</u> ) <u>thi</u> <u>supE/F<sup>+</sup></u> ( <u>traD36</u> <u>lacI<sup>Q</sup></u> <u>proA<sup>+</sup></u> <u>proB<sup>+</sup></u> <u>lacZ-del-M15</u> )	R. Hayward

**Table 2.2** Bacteriophages used in this work.

Notes

1. red3 is a missense mutation, gam210 and S7 are amber mutations and cI857 is a temperature sensitive mutation. pal571 indicates that the strain carries a 571 base pair palindrome.

2. Further information on these phages is provided in chapters 3,4 and 5.

**Table 2.3** Plasmids used in this work

Notes

1. Further information on these plasmids is provided in chapters 3 and 5.

Table 2.2

Phage	Notes	Description	Source
λDRL110	1,2	<u>red3</u> <u>gam210</u> <u>cI857</u> <u>ΔB</u> <u>pal571</u> <u>S7</u>	D. Leach
λJMC249	1,2	<u>Δspi6</u> <u>cI857</u> <u>S7</u>	F. Stahl
λDRL121	1,2	<u>red3</u> <u>gam210</u> <u>cI857</u> <u>ΔB</u> <u>S7</u>	D. Leach
λJMC082	1	<u>red3</u> <u>gam210</u> <u>chiA131</u>	F. Stahl
M13mp18	2		A. Robinson

Table 2,3

Plasmids	Notes	Description	Reference
pBR322		Ap <sup>R</sup> Tc <sup>R</sup>	Bolivar <u>et al.</u> (1977)
pUC18	1	Ap <sup>R</sup>	Yanisch-Perron <u>et al.</u> (1984)
pMB1	1	Ap <sup>R</sup>	Yoshimori <u>et al.</u> (1972)
pTP166	1	Ap <sup>R</sup>	Marinus <u>et al.</u> (1984)
pJL1	1	Ap <sup>R</sup>	This work

BBL top agar As for BBL agar but containing only 6.5g of Difco Agar per litre.

### 2.1.3 Media additives

Ampicillin (Beechem research laboratories). Ampicillin was added to media for the maintainance of Ap<sup>R</sup> plasmids in strains to a concentration of 100µg/ml.

### 2.1.4 General solutions

The following buffers were used in several procedures, all other buffers and solutions are listed under the specific procedure with which they were used.

TM buffer 10mM Tris; 10mM MgSO<sub>4</sub>. Adjusted to pH 7.5 with concentrated HCl. Autoclaved.

TE buffer 10mM Tris, 1mM EDTA. Adjusted to pH 7.5 with concentrated HCl. Autoclaved.

### 2.1.5 Solutions for bacterial storage

M9 salts (4x) Na<sub>2</sub>HPO<sub>4</sub>, 28g; KH<sub>2</sub>PO<sub>4</sub>, 12g; NaCl, 2g; NH<sub>4</sub>Cl, 4g; distilled water to 1 litre. Autoclaved.

Glycerol storage buffer 0.6 volumes of glycerol added to 0.4 volumes of M9 salts (4x). Autoclaved ,then 0.1ml of sterile MgSO<sub>4</sub> and 0.2ml of sterile CaCl<sub>2</sub> added.



### 2.1.6 Solutions for phage M13 titration

Phage buffer  $\text{Na}_2\text{HPO}_4$ , 7g;  $\text{KH}_2\text{PO}_4$ , 3g; NaCl, 5g; 0.1M  $\text{MgSO}_4$ , 10ml; 1% (w/v) gelatin, 1ml. Autoclaved.

### 2.1.7 Solutions for phenol extraction

Equilibrated phenol Distilled phenol was stored at 4°C under water in a light proof bottle, following the addition of 0.1% hydroxyquinoline and equilibrated with 1M Tris/HCl pH8 before use.

### 2.1.8 Solutions for ethanol precipitation

Sodium acetate solution(pH5.3) Sodium acetate solution (3M in acetate) was prepared by adding 0.19 volumes of sterile 3M Acetic acid to 0.81 volumes of sterile 3M sodium acetate.

tRNA (Boehringer Mannheim) Made up at 1mg/ml in sterile TE and stored at -20°C.

### 2.1.9 Solutions for DNA restriction

Buffers were made from sterile solutions and stored at 4°C.

Low salt restriction buffer (10x) 100mM Tris/HCl pH7.4; 100mM  $\text{MgSO}_4$ ; 10mM DTT.

High salt restriction buffer (10x) 1M NaCl; 500mM Tris/HCl  
pH 7.4; 100mM MgSO<sub>4</sub>.

SmaI restriction buffer (10x) 200mM KCl; 100mM Tris/HCl  
pH8; 100mM MgSO<sub>4</sub>; 10mM DTT.

Restriction enzymes Restriction enzymes used in this work  
are listed in table 2.4.

Final sample buffer (FSB) 0.1% bromophenol blue; 10%  
Ficol, 0.05% SDS in TE buffer.

#### 2.1.10 Solutions for DNA ligation

Buffers were made from sterile solutions and stored at -  
20°C.

Dilution buffer 50mM KCl; 10mM Tris/HCl pH7.5; 0.1mM EDTA;  
1mM DDT; 200µg/ml BSA; glycerol 50%.

Reaction buffer (10x) 500mM Tris/HCl pH7.8; 100mM MgCl<sub>2</sub>;  
200mM DDT; 500µg/ml BSA. ATP (disodium salt) was added to  
10mM just before use.

DNA ligase purchased from New England Biolabs.

#### 2.1.11 Solutions for detection of cloned inserts

Xgal (Anglian biochemicals). Made up at 24mg/ml in  
dimethylformamide. Stored at -20°C.

Table 2.4 Restriction enzymes used in this work.

Enzyme	Buffer	Suppliers
<u>EcoRI</u>	High salt	Boehringer Mannheim or Amersham International plc.
<u>SmaI</u>	<u>SmaI</u>	New England Biolabs
<u>AvaI</u>	Low salt	Bethseda research laboratories
<u>BamHI</u>	High salt	Boehringer Mannheim
<u>DpnI</u>	High salt	Boehringer Mannheim or Bethseda research laboratories
<u>MboI</u>	High salt	Bethseda research laboratories
<u>SstI</u>	High salt	Bethseda research laboratories
<u>PvuII</u>	Low salt	N.b.l. enzymes ltd.
<u>TaqI</u>	Low salt	Pharmacia
<u>HaeIII</u>	Low salt	Bethseda research laboratories

IPTG (Anglian biochemicals). Made up at 24mg/ml in sterile distilled water. Stored at  $-20^{\circ}\text{C}$ .

### 2.1.12 Solutions for Birnboim plasmid preparations

B/B buffer 25mM Tris/HCl pH8; 10mM EDTA; 0.9% glucose. Autoclaved.

Lysis solution 2mg/ml lysozyme in B/B buffer. Freshly prepared before use at  $0^{\circ}\text{C}$ .

Alkaline SDS solution 0.2M NaOH; 1% SDS. Freshly prepared at room temperature.

High salt solution Sodium acetate pH4.8 (3M in sodium) prepared by adding 0.4 volumes of sterile 5M acetic acid to 0.6 volumes of sterile 5M sodium acetate.

Low salt solution 0.1M sodium acetate adjusted to pH5.6 with glacial acetic acid.

### 2.1.13 Solutions for large scale plasmid preparations

25% sucrose solution 25% sucrose; 50mM Tris/HCl pH8. Autoclaved.

Lysozyme solution 20mg/ml lysozyme in sterile 0.25M Tris/HCl pH8. Freshly prepared at  $0^{\circ}\text{C}$  before use.

0.25M EDTA pH8 0.25M EDTA disodium salt adjusted to pH8 with glacial acetic acid. Autoclaved.

Lytic mix 50mM Tris/HCl pH8; 64.5mM EDTA; 0.1% w/v Triton (x100). Autoclaved.

Chloramphenicol purchased from Sigma chemical company. Used in solid form.

#### 2.1.14 Solutions for caesium chloride/ethidium bromide gradients

Ethidium bromide Made up at 10mg/ml in sterile TE buffer. Stored at 4°C in a light proof bottle.

CsCl/EtBr solution Prepared by adding 1ml of TE buffer and 0.1ml of ethidium bromide for every 1g of CsCl.

Butanol Butan-1-ol saturated with a solution containing 5M NaCl; 10mM Tris and 1mM EDTA adjusted to pH8 with concentrated HCl.

#### 2.1.15 Solutions for linear phage lambda DNA preparation

DNase (Sigma chemical company). Made up at 10mg/ml in sterile TE buffer and stored at -20°C.

RNase (Sigma chemical company). Made up at 10mg/ml in sterile TE buffer and then placed in a boiling water bath

for 10 minutes to denature any contaminating DNases (RNase is not denatured at 100°C). Stored at -20°C.

CsCl solutions 31.2%, 41.5%, 45.4% and 56.2% CsCl solutions made up w/w in sterile TM buffer.

Phage lysis buffer 20mM Tris; 100mM NaCl; 1mM EDTA; 0.002% Triton (x100).

Pronase (Sigma chemical company). Made up at 10mg/ml in sterile TE buffer and autodigested for 1 hour at 37°C to inactivate any contaminating nucleases. Stored at -20°C.

#### 2.1.16 Solutions for agarose gel electrophoresis

E. buffer 80mM Tris; 40mM sodium acetate; 2mM EDTA. Adjusted to pH8.2 with acetic acid.

Final sample buffer Described in section 2.1.9.

Ethidium bromide Described in section 2.1.14

#### 2.1.17 Solutions for Southern transfer of agarose gels

Denaturation buffer 1.5M NaCl; 0.5M NaOH.

Ammonium acetate solution 1M ammonium acetate adjusted to pH8 with NaOH.

### 2.1.18 Solutions for plaque transfer

Denaturation buffer Described in section 2.1.17

Neutralizing buffer 0.5M Tris/HCL pH7.4; 3M NaCl.

### 2.1.19 Solutions for nick translation

Nick translation buffer(4x) 210mM Tris/HCl pH7.5; 21mM MgCl<sub>2</sub>; 20µg/ml BSA. Prepared from sterile solutions and stored at -20°C.

dNTP buffer For a 400µl volume:- Nick translation buffer, 100µl; 2mM dGTP, 4µl; 2mM dATP, 4µl; 2mM dTTP, 4µl; 14M B-mercaptoethanol, 1µl, distilled water, 287µl. Stored in 20µl aliquots at -70°C.

DNA PolI dilution buffer (2x) 1mg/ml BSA, 2ml; 1M B-mercaptoethanol, 20µl; 2M ammonium sulphate, 100µl; 1M Tris/HCl pH7.5, 100µl. Made from sterile solutions and stored at -20°C.

DNase I dilution buffer PolI dilution buffer (2x), 1ml; glycerol, 1ml.

DNase I stock 1mg/ml DNase I (Boehringer Mannheim) was serially diluted in DNase I dilution buffer to a final concentration of  $2 \times 10^{-5}$  mg/ml DNase. Stored at -20°C.





DNA Pol I Purchased from Boehringer Mannheim.

### 2.1.20 Solutions for hybridisation

Denhardts solution (50x) Gelatin, 10g; polyvinyl pyrrolidine (MW 700,000), 1g; Ficoll 400, 1g; distilled water to 100ml. Stored at  $-20^{\circ}\text{C}$ .

1M Sodium phosphate buffer pH7 Prepared by adding 0.39 volumes of  $1\text{M NaH}_2\text{PO}_4$  to 0.61 volumes of  $1\text{M Na}_2\text{HPO}_4$ .

SSCP (10x)  $1.2\text{M NaCl}$ ;  $0.15\text{M Sodium citrate}$ ;  $0.2\text{M sodium phosphate buffer pH7}$ .

Calf thymus DNA Made up at  $5\text{mg/ml}$  in TE. Sonicated and phenol extracted. Stored at  $-20^{\circ}\text{C}$ .

Prehybridisation solution  $50\%$  formamide;  $4\text{xSSCP}$ ;  $5\text{x Denhardts solution}$ . Calf thymus DNA is denatured for 5 minutes in a boiling water bath and added to the prehybridisation solution at a concentration of  $10\mu\text{g/ml}$  just before use.

Hybridisation solution As for prehybridisation solution, but with the addition of  $3\%$  dextran sulphate.

SSC (20x)  $3\text{M NaCl}$ ;  $0.3\text{M sodium citrate}$ .

### 2.1.21 Solutions for polyacrylamide gel electrophoresis of DNA

30% Acrylamide solution 29% w/v acrylamide; 1% w/v bisacrylamide.

TBE (10x) 1M Tris; 0.83M boric acid; 10mM EDTA. pH8.3

10% AMPS 10% w/v ammonium persulphate in distilled water. Freshly prepared.

3% Acrylamide solution 30% Acrylamide solution, 1.5ml; TBE (10x), 1.5ml; 10% AMPS, 0.13ml; distilled water to 15ml.

10% Acrylamide solution 30% Acrylamide solution, 5ml; TBE (10x), 1.5ml; 10% AMPS, 0.13ml; distilled water to 15ml.

Stacking gel 30% Acrylamide solution, 5ml; TBE (10%), 1ml; 10% AMPS, 0.16ml; distilled water to 10ml.

TBE loading buffer 0.5xTBE; 0.125M EDTA; 0.1% bromophenol blue; 50% v/v glycerol.

### 2.1.22 Solutions for purification of plasmid DNA for sequencing

Sephacryl column buffer 0.3M sodium acetate; 0.1% SDS in TE buffer.

### 2.1.23 Solutions for plasmid sequencing

Solutions were made up in double distilled water.

Denaturing buffer 0.2mM EDTA pH8; 0.2M NaOH. Autoclaved.

Ammonium acetate solution 2M ammonium acetate. pH adjusted to 4.5 with glacial acetic acid. Sterile filtered.

Annealing buffer (10x) 70mM Tris/HCl pH7.5; 70mM MgCl<sub>2</sub>; 300mM NaCl; 100mM DTT and 1mM EDTA pH8. Sterile filtered and stored at -70°C.

Oligonucleotide primer 17mer sequencing primer 5'd(GTAAAACGACGGCCAGT)3' (2.5 pmol/μl) was kindly donated by Dr J. Beggs and was originally purchased from New England Biolabs.

Deoxynucleotides (Boehringer Mannheim). 10mM stock solution, 0.5mM working solution. Made up in sterile double distilled water.

Dideoxynucleotides (Boehringer Mannheim). 10mM stock solution, 0.5mM working solution. Made up in sterile double distilled water.

Sequencing reaction mixes The sequencing reaction mixes were made up as shown in table 2.5. The reaction mixes were kindly supplied by G. Cowan and stored at  $-70^{\circ}\text{C}$ .

Chase solution 0.125mM dNTP (all four). Chase solution was kindly supplied by E. Okely and stored at  $-70^{\circ}\text{C}$ .

Formamide dye mix 98% deionised formamide (w/w); 10mM EDTA pH8; 0.2% bromophenol blue (w/v); 0.2% xylene cyanol (w/v). Stored at  $4^{\circ}\text{C}$ .

#### 2.1.24 Solutions for polyacrylamide sequencing gels

TBE (10x) Described in section 2.1.21

40% Acrylamide solution 38% (w/v) acrylamide; 2% (w/v) bisacrylamide.

0.5 TBE gel mix 40% Acrylamide solution, 150ml; 10x TBE, 50ml; urea, 460g; distilled water to 1 litre.

2.5 TBE gel mix 40% Acrylamide solution, 150ml; 10x TBE, 250ml; urea, 460g; sucrose, 50g; bromophenol blue, 50mg; distilled water to 1 litre.

25% AMPS 25% ammonium persulphate (w/v) in sterile distilled water. Freshly prepared.

Table 2.5 The composition of the sequencing reaction mixes.

Nucleotide (working solution)	T mix $\mu\text{l}$	C mix $\mu\text{l}$	G mix $\mu\text{l}$	A mix $\mu\text{l}$
dTTP	2	40	40	40
dCTP	40	4	40	40
dGTP	40	40	2	40
ddTTP	50	-	-	-
ddCTP	-	10	-	-
ddGTP	-	-	12	-
ddATP	-	-	-	10
10x annealing buffer	20	20	20	20
H <sub>2</sub> O	48	86	86	50

Fix 10% methanol; 10% glacial acetic acid.

## 2.2 Methods

All media, solutions and enzymes are described in section 2.1.

### 2.2.1 Storage and growth of bacteria

Bacteria were stored at 4°C on L plates. For longer term storage, 0.5ml of a stationary phase culture was added to 2ml of glycerol storage buffer in a screw top bijoux bottle and stored at -20°C. Overnight cultures were grown by inoculating a colony into L broth and shaking at 37°C, except where otherwise stated.

### 2.2.2 Titration of phage lambda

A plating culture was prepared by diluting an overnight culture of bacteria 10 fold in L broth p/c and growing with shaking at 37°C to  $2 \times 10^8$  cells/ml ( $OD_{650}$  0.45), then adding an equal volume of TM buffer. Fresh plating cultures were prepared daily. Lambda phage stocks were serially diluted in TM buffer and 0.1ml of an appropriate dilution was added to 0.25ml of bacterial plating culture. The mixture was left at room temperature for 10 minutes to allow the phage to adsorb. 2.5ml of molten BBL top agar (cooled to 46°C) was then added and the mixture poured onto the surface of a BBL plate. Plates were incubated at 37°C overnight.

### 2.2.3 Titration of phage M13

All cultures of JM101 were grown with slow shaking to



avoid shearing of the pili.

An overnight of JM101 was diluted 100 fold in L broth and grown to  $2 \times 10^8$  cells/ml. This culture could be used directly for plating M13 on. M13 was diluted serially in phage buffer and 0.1ml was added to 0.5ml of a plating culture of JM101. The phage were allowed to adsorb for 20 minutes at room temperature. 2.5ml of BBL top agar (cooled to  $46^\circ\text{C}$ ) was added and the mixture poured onto the surface of a BBL plate. Plates were incubated at  $37^\circ\text{C}$  overnight.

#### **2.2.4 Production of phage lambda stocks by plate lysates**

A single plaque was picked using a pasteur pipette into 1ml of TM buffer, 20 $\mu$ l of chloroform was added and the mixture whirlmixed. This mixture was then left for an hour or more to allow the phage to diffuse from the agar. 0.3ml of this phage suspension was added to 0.25ml of a bacterial plating culture and allowed to adsorb for 10 minutes at room temperature. 2.5ml of BBL top agar was then added and the mixture poured onto the surface of a BBL plate. This plate was incubated for 4 to 10 hours until the lawn was approaching confluent lysis (plaques distinct, but touching one another). 5ml of TM buffer was then poured onto the surface and the plate left overnight at  $4^\circ\text{C}$ . The following day the TM buffer was decanted, 0.2ml of chloroform added and the mixture whirlmixed. Chloroform and debris were removed by centrifugation at 4.5krpm in a Centaur-2 bench centrifuge for 10 minutes. The supernatant was collected, titred and stored at  $4^\circ\text{C}$ .

This method was also suitable for the growth of M13 phage stocks using 0.5ml of a JM101 plating culture.

#### 2.2.5 Production of phage lambda stock by induction of temperature sensitive lysogens

All lysogens used to prepare phage stocks were lysogenic for cI857 phage carrying unsuppressed S7 mutations and therefore appropriate for use with the following procedure.

An overnight culture, which had been grown at 30°C, was diluted 100 fold and grown to  $2 \times 10^8$  cells/ml at 30°C. The flask containing the culture was placed in a 60°C waterbath until the temperature of the culture reached 43°C, then transferred to a 43°C waterbath and left for 15 minutes. The induced culture was shaken vigorously for 4 hours. The cells were then spun down at 8krpm for 8 minutes in a Sorvall centrifuge (GSA rotor), resuspended in 0.1 volumes of TM buffer and chloroform (10ml/litre) added. This suspension was gently homogenized, by swirling on a rotary shaker at 4°C overnight, then spun down at 4.5krpm in a Centaur-2 bench centrifuge to remove the chloroform and cell debris. The supernatant was collected, titred and stored at 4°C.

#### 2.2.6 Production of phage stocks by one step liquid lysates

This procedure is similar to a conventional liquid lysis, but phage are allowed only one or two rounds of lysis in the host bacteria. The procedure is

particularly useful for the growth of palindromic phage in hosts which are not recBC sbcB sbcC, to minimise the proportion of phage which have deleted the palindrome.

(i) Growth of phage with an unsuppressed S7 mutation.

An overnight culture was diluted 100 fold in L broth p/c and grown shaking at 37°C to  $2 \times 10^8$  cells/ml. The cells were then spun down at 8krpm for 8 minutes in a Sorvall centrifuge (GSA rotor) and resuspended in 0.1 volumes of TM buffer. After starving the cells by shaking in TM for 30 minutes at 37°C, phage were added at a multiplicity of infection (m.o.i) of 5 and absorbed to the bacteria at room temperature for 30 minutes. The mixture was then diluted 10 fold into L broth (prewarmed to 37°C) and shaken for 2 hours, after which time the cells were spun down at 8krpm for 8 minutes in a Sorvall centrifuge (GSA rotor) and resuspended in 0.025 volumes of TM buffer. RNase and DNase were each added to a concentration of 50µg/ml, together with chloroform (10ml/litre) and the suspension was gently homogenized, by swirling on a rotary shaker overnight at 4°C. The cell debris and chloroform were spun down at 4.5krpm in a Centaur-2 bench centrifuge and the supernatant decanted, titred and stored.

This method was adapted to produce fully dam modified lambda phage stocks by using strain JL28 and diluting the bacteria and adsorbed phage into L broth with the inducer IPTG added to a concentration of 0.24g/litre (the rationale behind this procedure is explained in chapter 5).

(ii) Growth of S<sup>+</sup> phage or phage with a suppressed S7

## mutation

The procedure used was the same as that given for phage with an unsuppressed S7 mutation up to the adsorption of phage. Following adsorption the mixture was diluted 100 fold into L broth (prewarmed to 37°C) and shaken for 90 minutes at 37°C. Chloroform was then added to a concentration of 2ml/litre and the mixture swirled on ice for 15 minutes. Cell debris and chloroform were removed by centrifugation at 8krpm for 8 minutes (Sorvall centrifuge, GSA rotor) and the phage stock decanted, then concentrated 50 fold by polyethylene glycol (PEG) precipitation as described below.

### 2.2.7 PEG precipitation of phage

RNase and DNase were each added to the clarified phage lysate to a concentration of 1µg/ml and digestion was allowed to proceed for an hour at room temperature. Sodium chloride was then added to 40g/litre, followed by solid PEG 6,000 to a concentration of 10% w/v. The PEG was dissolved by swirling the mixture at room temperature for several minutes and the solution was then left at 4°C for at least an hour. The precipitated phage were spun down at 10krpm for 10 minutes (Sorvall centrifuge, GSA rotor) and resuspended in 0.02 volumes of TM buffer by gentle shaking at 4°C on a rotary shaker for an hour. Debris was removed by centrifugation at 4.5krpm for 10 minutes (Centaur-2 bench centrifuge) and the suspension decanted, titred and stored.

### 2.2.8 Phenol extraction

Phenol extractions were carried out in 30ml Corex tubes or Eppendorf tubes. An equal volume of equilibrated phenol was added to the DNA solution and mixed by inverting several times. After leaving to stand for a few minutes the two layers were separated by centrifugation for 5 minutes at 5krpm in a Sorvall centrifuge (SS34 rotor) or at maximum speed in an Eppendorf centrifuge. The bottom layer (phenol) was then removed with any material at the interface and discarded. The top layer was either dialysed against TE buffer or ethanol precipitated to remove any remaining phenol.

### 2.2.9 Ethanol precipitation

Ethanol precipitations were carried out in 30ml Corex tubes or Eppendorf tubes. 0.1 volumes of sodium acetate solution pH5.3 were added to 0.9 volumes of DNA dissolved in TE buffer, followed by 2 volumes of ethanol and the tube contents mixed by inversion. When precipitating small amounts of DNA as in the case of the lambda supercoils taken from a CsCl/EtBr gradient, tRNA was added to a concentration of 5µg/ml to maximise recovery of precipitated DNA. The DNA was left precipitating at -20°C for at least an hour then pelleted by centrifugation for 10 minutes at 10krpm in a Sorvall centrifuge (SS34 rotor) or at maximum speed in an Eppendorf centrifuge. The pelleted DNA was dried in a vacuum desiccator.

#### 2.2.10 DNA restriction

A list of the restriction enzymes used is given in table 2.4. Typically reactions were carried out in a total volume of 10-15 $\mu$ l. 0.1 volumes of the appropriate 10x restriction buffer were added to 0.9 volumes of DNA in TE buffer, followed by an excess of enzyme to ensure complete digestion. The samples were incubated for 2 hours at 37°C and the reaction stopped by heating at 70°C for 10 minutes or, if the samples were to be directly used in agarose gel electrophoresis, by the addition of 0.25 volumes of final sample buffer.

#### 2.2.11 DNA ligation

In cloning reactions 0.1 $\mu$ g of vector was used and 3 fragment molecules were added for every vector molecule. Reactions were carried out in a total volume of 15 $\mu$ l. 0.1 volumes of 10x ligase reaction buffer were added to 0.9 volumes of DNA in TE buffer, followed by approximately 40 units of ligase. Reactions were incubated at 12°C overnight.

#### 2.2.12 Transformation (Mandel and Higa, 1975)

A single colony was picked and grown to  $2 \times 10^8$  cells/ml in 20ml of L broth. This culture was cooled for 15 minutes on ice and spun down for 5 minutes at 4.5krpm in a Centaur-2 bench centrifuge. Following resuspension in 10ml of ice cold 100mM MgCl<sub>2</sub>, the cells were immediately respun for 5 minutes and resuspended in 10ml of ice cold 100mM CaCl<sub>2</sub>, then left on ice for 30 minutes. The cells were

then spun down again and resuspended in 1ml of ice cold 100mM CaCl<sub>2</sub>. 200µl aliquots were dispensed into pre-cooled small test-tubes and approximately 50ng of DNA was added per aliquot. The mixture was left on ice for 30 minutes, then heat shocked in a 42°C waterbath for 2 minutes and returned to ice for a further 5 minutes. If a plasmid transformation was being performed, 0.5ml of L broth was added and the cells were shaken at 37°C to allow expression of the antibiotic resistance gene of the plasmid. 50µl aliquots of this culture were then spread on the surface of the selective antibiotic plates. If an M13 transformation was being performed then the transformed cells were treated as described in the next section.

#### 2.2.13 Detection of cloned inserts in puc18 and M13mp18 vectors following transformation

To detect cloned inserts in puc18, 20µl of IPTG and 30µl of Xgal were spread on the surface of the selective plates before spreading the transformed cells.

To detect cloned inserts in M13mp18 the transformed cells were added to 0.5ml of a fresh JM101 culture of  $2 \times 10^8$  cells/ml. 20µl of IPTG and 30µl of Xgal were added to 3ml of BBL top agar in a large test-tube, followed by the transformed cells/JM101 mix. The mixture was then vortexed to ensure the IPTG and Xgal were evenly distributed and poured onto the surface of a BBL plate. Plates were incubated at 37°C overnight.

Plasmids or phage with inserts gave white colonies or

plaques respectively on the IPTG/Xgal indicator plates, those without gave blue.

#### 2.2.14 Birnboim plasmid preparation (Birnboim and Doly, 1979)

1.5ml of an overnight culture of the plasmid containing strain, grown in L broth with the appropriate antibiotic, was spun down in an Eppendorf centrifuge for 1 minute at maximum speed. The supernatant was removed and the cells resuspended in 100µl of lysis solution, then put on ice for 30 minutes. 200µl of alkaline SDS solution was added and the cells were returned to ice for five minutes, before adding 150µl of high salt solution. Following this addition, the tube contents were mixed by inversion, then left on ice for a further 60 minutes. The mixture was then centrifuged for 1 minute at maximum speed in an Eppendorf centrifuge and 400µl of supernatant decanted. After the addition of 1ml of ethanol, the supernatant was left at -20°C for 30 minutes, then centrifuged for 5 minutes (maximum speed, Eppendorf centrifuge). The supernatant was removed and the pellet dissolved in 100µl low salt solution, followed by the addition of 200µl of cold ethanol. After leaving to precipitate at -20°C for 30 minutes the DNA pellet was spun down as before, dried in a vacuum desiccator and resuspended in 50µl of TE buffer.

#### 2.2.15 Large scale plasmid preparation (Clewell and Helinski, 1969).

##### (i) Plasmid amplification



An overnight culture, grown in L broth with the appropriate antibiotic (ampicillin for all plasmids used in this work) was diluted 50 fold into 500ml of L broth, containing antibiotic and 0.2% glucose and grown shaking at 37°C to an OD<sub>590</sub> of 1. Chloramphenicol was then added to a concentration of 150µg/ml and the culture was shaken for a further 16 hours.

(ii) Plasmid DNA extraction

Cells were harvested by centrifugation at 8krpm for 8 minutes (Sorvall centrifuge, GSA rotor) and resuspended in 16.5ml of ice cold 25% sucrose solution. After the addition of 3.9ml of lysozyme solution the suspension was left on ice for 10 minutes, followed by the addition of 6.5ml of 0.25M EDTA pH8 and a further 10 minute incubation on ice. 26.5ml of lytic mix was then added and the cells were left on ice for 20 minutes to allow lysis to occur. This 'crude lysate' was spun at 18krpm for 30 minutes (Sorvall centrifuge, SS34 rotor) to pellet the chromosome and cell membrane. The supernatant was decanted and phenol extracted once or twice. The DNA was then ethanol precipitated twice and put down two caesium chloride/ethidium bromide gradients as described in section 2.2.18.

2.2.16 Lambda supercoil preparation (Adapted from Reuben et al.,1974)

An overnight culture was diluted 100 fold in 100ml of L broth p/c and grown with shaking at 37°C to  $2 \times 10^8$  cells/ml. The cells were then spun down at 8krpm for 8

minutes (Sorvall centrifuge, GSA rotor) and resuspended in 10ml of TM buffer. Following starvation of the cells by shaking for 30 minutes in TM at 37°C, a mixture of two lambda phage were added at a m.o.i of 2 for each phage (see chapters 4 and 5 for details). After allowing the phage to adsorb at room temperature for 30 minutes, the mixture was diluted 50 fold into L broth prewarmed to 37°C. The culture was agitated vigorously at 37°C for a specified length of time (see chapters 4 and 5). The cells were then cooled in an ice/water bath to prevent further growth and harvested by centrifugation at 8krpm for 8 minutes (Sorvall centrifuge, GSA rotor). The cells were washed twice with ice cold L broth to remove unadsorbed phage and finally resuspended in 3.3ml of ice cold 25% sucrose solution. The DNA extraction procedure then followed was the same as that described for large scale plasmid preparation, except the volumes of solutions used were scaled down 5 fold in accordance with the initial culture volume of 100ml as opposed to 500ml.

#### **2.2.17 M13 replicative form preparation**

A fresh M13 plaque and the surrounding cells were picked with a pasteur pipette into 1ml of L broth and grown overnight shaking slowly at 37°C. 0.5ml of this overnight was added to 500ml of a fresh JM101 culture of  $2 \times 10^{-8}$  cells/ml. This mixture was grown shaking slowly at 37°C for 4 hours, then the cells were harvested by centrifugation at 8krpm for 8 minutes and resuspended in 16.5ml of ice cold 25% sucrose solution. The DNA

extraction procedure then followed was the same as that described for large scale plasmid preparation.

#### 2.2.18 Purification of supercoiled DNA on caesium chloride / ethidium bromide (CsCl/EtBr) gradients

Usually supercoiled DNA was put down two gradients, this removes the vast majority of chromosomal contamination. When lambda supercoil preparations were being performed, there was usually insufficient lambda supercoiled DNA to see a band under long wave UV light. For this reason, most of the lambda supercoil experiments were performed either with strains which contained a plasmid (pBR322) or more often supercoiled plasmid DNA from a large scale plasmid preparation was added to the extracted DNA before it was put down the gradients. For some lambda supercoil experiments (those described in chapter 4) this was not done, the supercoils from these experiments were put down only one gradient (gradient 2) and the material was removed from the part of the gradient where the supercoils would be expected to run.

##### (i) Gradient 1

Pellets of DNA from ethanol precipitated supercoil preparations were dried in a vacuum desiccator and dissolved in 8ml of TE. 8g of CsCl was dissolved in this solution, then 0.8ml of ethidium bromide solution added. This solution was put in a 11.5ml Sorvall polyallomer tube and the tube was filled to the base of the neck with CsCl/EtBr solution. Tubes were balanced in pairs to within 0.05g and sealed with a Sorvall crimp sealer. The tubes

were spun in a Sorvall Ti50 rotor in a Sorvall ultracentrifuge for 48 hours at 38Krpm and 20°C. After centrifugation the tubes were illuminated with long wave UV which revealed the presence of two bands. The upper band was chromosomal and relaxed DNA, the lower band was supercoiled DNA and was removed using a syringe and needle.

(ii) Gradient 2

The supercoiled DNA removed from gradient 1 was put down a second gradient. The CsCl solution sample containing the supercoiled band was put in a 6ml Sorvall polyallomer tube and this tube was then filled to the base of the neck with CsCl/EtBr solution. Tubes were balanced and crimp sealed as before, then spun for 16 hours at 20°C in a Sorvall TV865 rotor in a Sorvall ultracentrifuge at 50krpm. After centrifugation, the tube was illuminated with long wave UV. Usually a faint trace of relaxed band could be seen. The supercoiled band was removed as before and the ethidium bromide butanol extracted.

(iii) Butanol extraction

An equal volume of butan-1-ol was added to the CsCl/EtBr solution and mixed by inverting several times. The mixture was left standing until the two layers separated. The top layer was then removed. Three or four butanol extractions were performed, until the butanol layer was no longer pink.

(iv) Ethanol precipitation of gradient DNA

Following butanol extraction, 2 volumes of TE buffer and 6 volumes of ethanol were added to the CsCl solution

and the mixture was left at  $-20^{\circ}\text{C}$  for an hour. The DNA was then spun down at 10krpm for 10 minutes (Sorvall centrifuge, SS34 rotor) and the DNA pellet resuspended in 2ml of TE buffer. A second ethanol precipitation was performed as described in section 2.2.9 and the resulting DNA pellet was dried in a vacuum desiccator. The dried DNA pellet was dissolved in 500 $\mu\text{l}$  of TE in the case of plasmid and M13 replicative form preparations, but in only 60 $\mu\text{l}$  of TE for lambda supercoils. The concentration of DNA was then estimated by running 10 $\mu\text{l}$  on a 1% agarose gel against a standard DNA of known concentration. Lambda supercoils were usually dialysed against TE buffer at  $4^{\circ}\text{C}$  in a BRL mini dialysis apparatus for 1 hour to remove any contaminating small molecules.

### 2.2.19 Preparation of linear phage lambda DNA

#### (i) Purification of phage on a step gradient

RNase and DNase were each added to a concentration of 10 $\mu\text{g}/\text{ml}$  to a phage suspension which contained at least  $10^{12}$  phage and left to digest for an hour at room temperature. 1.5ml of the 31.2% CsCl solution was put in a 14ml MSE polycarbonate tube. This was gently underlayered with the 45.4% CsCl solution using a pasteur pipette and these two layers were then underlayered with the 56.2% CsCl solution. The phage sample was gently added on top of the CsCl layers and the tube filled to within 2mm from the top. Tubes were balanced to within 0.05g and spun in an MSE titanium 6x14ml swing out rotor in an MSE superspeed 65 centrifuge for 2 hours at 33krpm and  $20^{\circ}\text{C}$ . Two bands

could be seen in visible light, the top band was protein and debris, the lower band was the phage and was collected using a syringe and needle.

(ii) Further purification of phage on an equilibrium gradient

The phage band from the step gradient was mixed with the 41.5% CsCl solution and put in a Sorvall 6ml polyallomer tube. Tubes were balanced in pairs to within 0.05g, crimp sealed, then spun for 16 hours at 50krpm and 20°C in a Sorvall TV865 rotor in a Sorvall ultracentrifuge. The phage band was collected from the side using a syringe and needle after centrifugation.

(iii) Preparation of phage DNA

The CsCl solution containing the phage band was dialysed at 4°C against three changes of TE (total volume 5 litres) to remove the CsCl. Pronase was then added to concentration of 1mg/ml and the solution was dialysed against phage lysis buffer (2 litres) for 2 hours at 37°C. The dialysed solution was phenol extracted three times and then dialysed against three changes of TE (total volume 5 litres) to remove all traces of phenol. Following dialysis the DNA concentration was estimated by running 10µl on a 1% agarose gel against a standard DNA of known concentration.

#### 2.2.20 Agarose gel electrophoresis

Two sizes of horizontal agarose gels were used, midi gels ( dimensions 14cm x 11cm x 0.65cm) and maxi gels (dimensions 25cm x 20cm x 0.6cm). All gels were 0.7%

agarose. Midi gels were prepared by adding 0.7g of agarose to E buffer in a total volume of 100ml (for maxi gels triple quantities were used) and dissolving the agarose by simmering the mixture in a microwave oven. The molten agarose solution was cooled to 50°C before pouring into a perspex mould with a 14 tooth slot former (20 tooth for a maxi gel). The gel was allowed to set and then placed into a BRL horizontal system for submerged gel electrophoresis with 900ml of E buffer (2.5l for maxi gels) and the slot former removed. 1 volume of final sample buffer was added to 4 volumes of the DNA samples to be analysed. The DNA samples were then carefully pipetted into the gel slots using a micropipetter. Gels were run at 1.5 volts/cm overnight and stained by adding ethidium bromide to the E buffer to a concentration of 0.5µg/ml. After staining for an hour, gels were washed in water and examined in long wave UV light on a C-62 BLAK-RAY transilluminator (Ultraviolet products incorporated). Photographs of gels were made on Ilford H5 film using a red filter and a 5 second exposure.

#### 2.2.21 Southern transfer of agarose gels

The procedure used was a Smith and Summers (1980) modification of the original Southern (1975) method.

The portion of the agarose gel containing the bands was cut out and placed in 0.25M HCL. This partially hydrolysed the DNA. The gel was then rinsed in water and denaturation solution added. After 30 minutes in denaturation solution the gel was again rinsed and

neutralized for an hour in ammonium acetate solution. The neutralized gel was placed on a perspex slab and covered with a nitrocellulose filter (Schleicher and Schull, pore size 0.45 $\mu$ m) cut to the same size as the gel, which had previously been soaked in the ammonium acetate solution. Any trapped air bubbles were removed by gentle rolling with a 10ml pipette. Four sheets of blotting paper cut to the same size were soaked in the ammonium acetate solution and placed on the nitrocellulose filter one at a time, removing air bubbles as before. A 2cm thick stack of blotting paper of the same size was placed on top and the apparatus was wrapped in Saran wrap. A weighted perspex sheet was then placed on top and the gel was left to transfer overnight. The following day, the nitrocellulose filter was removed from the surface of the gel and the gel discarded. The filter was washed in 2xSSC for 5 minutes to remove salt, dried at 37°C between two sheets of blotting paper for 15 minutes and then baked in a vacuum oven at 80°C for 90 minutes.

#### 2.2.22 Plaque transfer (Benton and Davis, 1977)

Plaques to be investigated were picked using a toothpick and stabbed into a lawn of bacteria in BBL top agar in a grid formation. A very dry BBL plate was used to ensure the top agar adhered well. The plate was then incubated at 37°C overnight. In the morning a nitrocellulose filter (Amersham Hybon-D, pore size 0.45 $\mu$ m) was laid on the surface of the agar and allowed to become wet. Care was taken to ensure that the filter and plate



had an orientation mark for later reference. The wet filter was peeled off and laid plaque side up on a 2cm pad of blotting paper, soaked in denaturation solution, for 2 minutes. The filter was then dropped in a beaker containing neutralization solution and left for 1 minute. The neutralized filter was washed in 2xSSC for 5 minutes, dried at 37°C and baked in a vacuum oven at 80°C for 90 minutes.

#### 2.2.23 Nick translation (Maniatis et al.,1975 and Rigby et al.,1977)

10 to 15 $\mu$ Ci of [ $\alpha$ <sup>32</sup>P] dCTP was used per reaction. 20 $\mu$ l of dNTP buffer was added to the [ $\alpha$ <sup>32</sup>P] dCTP, followed by 5 $\mu$ l of DNA (about 0.5 $\mu$ g). 1 $\mu$ l of DNase I stock and 0.5 $\mu$ l of polymerase I (5 units/ $\mu$ l) were then added to the reaction mixture and the mixture incubated at 15°C for 3 hours. The reaction was stopped by increasing the volume to 200 $\mu$ l with distilled water and phenol extracting. The phenol was back extracted once with distilled water. The unincorporated nucleotides were then removed by putting the sample down a Sephadex G-50 (fine) column 13cm long with a diameter of 0.75cm. Ten drop fractions were collected and monitored with a mini monitor. The fractions corresponding to the first peak of radioactivity were pooled, other fractions were discarded. The radioactive DNA sample is referred to as a 'probe'.

#### 2.2.24 Hybridisation

The baked nitrocellulose filters were wetted in 2xSSC

and placed in a plastic bag with the pre-hybridisation solution (20ml for filters from agarose gels, 10ml for plaque hybridisations). Air was removed from the plastic bag, which was then sealed and shaken for 1 hour or more at 37°C. After prehybridisation, the filter was placed in a fresh plastic bag and hybridisation solution added (10ml or 20ml, as before). The radioactive probe was placed in a boiling waterbath for 5 minutes to denature it and added to the hybridisation solution. The air was removed from the plastic bag and the bag sealed. Hybridisation was carried out overnight, shaking at 37°C. Following hybridisation the filter was washed for 45 minutes in 3 changes of 2xSSC, 0.1% SDS (total volume 1 litre) at room temperature, then for 60 minutes in 2 changes of 1xSSC, 0.1% SDS (total volume 1 litre) at 37°C. The SDS was then rinsed off with two 5 minute washes in 500ml of 2xSSC and the filter was blotted dry, wrapped in Saran wrap and autoradiographed.

#### 2.2.25 Autoradiography

Autoradiography was carried out at -70°C in Cronex (Du Pont) cassettes fitted with 'xtra-life' intensifying screens, using Cronex (Du Pont) grade 4 X-ray film preflashed to an OD<sub>540</sub> of 0.15. Exposure time varied, but was usually 1 to 8 hours for filters and 1 to 3 days for sequencing gels.

#### 2.2.26 Densitometry

The amount of material present in bands was estimated

using a Vitratron densitometer with a 100 $\mu$ m slitpiece and a grey (U60) filter attached to a chart recorder. Peaks were integrated by tracing on a Summagraphics magnetic data tablet, attached to a Ferranti-Cetec digitiser and an Olivetti P<sub>640</sub> microcomputer, programmed by Dr A. Coulson to perform area calculations. Several exposures of film were analysed to ensure that the response was approximately linear.

#### 2.2.27 Polyacrylamide gel electrophoresis of DNA (Maniatis *et al.*, 1985).

A vertical 3-10% gradient polyacrylamide gel was used for the separation of DNA fragments of only a few hundred base pairs or less.

3% and 10% acrylamide solutions were made up and degassed. 10 $\mu$ l of TEMED was added to each and the solutions were poured into a 2 chambered linear gradient maker. The acrylamide solutions were poured from the gradient maker into a glass sandwich (dimensions 20cm x 14cm x 0.1cm) with perspex spacers sealed with 0.5% agarose. The gradient was overlaid with butan-2-ol and left to polymerise for 2 hours. A stacking gel solution was prepared and degassed. The butan-2-ol was decanted from the surface of the polymerised gradient gel and the gel surface was rinsed with distilled water. 10 $\mu$ l of TEMED was added to the stacking gel and this was used to overlay the gradient gel. A 13 tooth slot former was inserted and the stacking gel was left for 30 minutes to polymerise. After polymerisation the slot former and the bottom spacer

were removed and the gel attached to a perspex gel apparatus with 400ml of TBE buffer in each reservoir. The gel slots were washed out. The DNA samples to be analysed were ethanol precipitated, dried and resuspended in 10 $\mu$ l of TE and 5 $\mu$ l of TBE loading buffer was added. The samples were loaded using a Hamilton syringe and the gel was run at 12.5v/cm for 30 minutes, then at 4v/cm for 18 hours. After the gel had run, the two glass plates were separated, leaving the gel attached to one. The gel was stained for a few minutes in 3 $\mu$ g/ml ethidium bromide, washed with water and photographed (the gel was removed from the glass plate before photographing).

#### 2.2.28 Purification of plasmid DNA for sequencing

Plasmid DNA, isolated by the large scale plasmid preparation procedure (section 2.2.15) and purified on a CsCl/EtBr gradient, was further purified before sequencing on a Sephacryl S1,000 (superfine) column 45cm long and 1cm in diameter. The column was attached to a Uvicord SII 2238 compact single beam UV monitor and a LKB 2211 superrac fraction collector. The UV monitor was attached to a chart recorder which plotted the absorbance at 206nm of the solution passing through it. Fractions corresponding to the first peak of absorbance were pooled. (There was usually a large second peak of absorbance, containing material which couldn't be seen on a gel and may have been ethidium bromide not removed by butanol extraction). 2 volumes of ethanol were added to the pooled fractions and they were precipitated at -20°C for 1 hour, spun down at

10krpm for 10 minutes (Sorvall centrifuge, SS34 rotor), resuspended in TE and ethanol precipitated again. Following the second ethanol precipitation, the dried DNA pellets were resuspended in TE and the concentration of the DNA estimated by running a sample on an agarose gel against a known standard.

2.2.29 Plasmid DNA sequencing (Chen and Seeburg, 1985; Original dideoxy sequencing method devised by Sanger et al., 1977).

(i) Denaturation of plasmid DNA

2 $\mu$ g of DNA was dried down, resuspended in 40 $\mu$ l of denaturing buffer and allowed to stand at room temperature for 5 minutes. 4 $\mu$ l of neutralizing ammonium acetate solution was then added and the DNA was precipitated by adding 2 volumes of ethanol and leaving at -70°C for an hour. The DNA was spun down in an Eppendorf centrifuge at 4°C for 10 minutes and then washed in 1ml of 70% ethanol, centrifuged and dried under vacuum.

(ii) Annealing of primer

1 $\mu$ l of 17mer oligonucleotide primer (2.5 pmol/ $\mu$ l), 1.5 $\mu$ l of 10x annealing buffer and 11.5 $\mu$ l of sterile double distilled water were mixed and then used to dissolve the denatured plasmid DNA. 1 $\mu$ l of [<sup>35</sup>S] dATP (10 $\mu$ Ci/ $\mu$ l) was then added and the mixture incubated at 37°C for 15 minutes.

(iii) Sequencing reactions

Four Eppendorf tubes were labelled T, C, G and A and 2 $\mu$ l of the corresponding dNTP/ddNTP mix was placed on one side

of each tube. 2 $\mu$ l of Klenow fragment (1U/ $\mu$ l) was added to the annealing reaction after the 15 minute incubation. 3 $\mu$ l of the annealing/Klenow mixture was then placed on the opposite side of the Eppendorf tubes and the two drops were added to each other by spinning the tubes for a few seconds in an Eppendorf centrifuge. This ensured that all reactions started at the same time. The reactions were incubated at 37°C for 20 minutes, then 1.5 $\mu$ l of chase solution was added to each tube, followed by a further incubation of 15 minutes at 37°C. The samples were dried under vacuum and resuspended in 4 $\mu$ l of formamide dye mix. Before loading on a gel the samples were denatured by placing in a boiling water bath for 3 minutes, then rapidly cooling on ice.

### 2.2.30 Polyacrylamide gel electrophoresis for sequencing (Biggin et al.,1983)

7ml of 2.5 TBE gel mix and 35ml of 0.5 TBE gel mix were placed in separate glass beakers. 14 and 70 $\mu$ l of 25% AMPS respectively were added to the two gel mixes, followed by 14 and 70 $\mu$ l of TEMED. Immediately following the addition of TEMED 4ml of the 0.5 gel mix was taken up in a 10ml pipette followed by 6ml of the 2.5 gel mix. A gradient was formed by introducing 5 air bubbles into the pipette. The gel was then poured carefully down the side of a glass sandwich (dimensions 40cm x 20cm x 0.05cm) with perspex side spacers, sealed with Sellotape. The gel was topped up by forcefully flushing 0.5 TBE gel mix across the top of the gel and a flat sided spacer was inserted to

form a single preparative well. The top of the gel was covered with Saran wrap and the gel was laid flat and allowed to set for at least 30 minutes. The polymerised gel was attached to a perspex gel apparatus, after first removing the Saran wrap and Sellotape. The top spacer was removed and the large well cleaned out. A sharks tooth comb was then inserted so that the teeth just touched the surface of the gel. 1 $\mu$ l aliquots of the DNA samples were loaded between the teeth of the comb with a drawn out micropipette tip. The samples were loaded in the order T,C,G,A and a solo T track was loaded at the left hand side of the gel for later orientation. The gel was run at 50v/cm. After the dye from the first set of DNA samples had run to the end of the gel (approximately 2 hours later) the same set of samples were loaded in the neighbouring tracks and the gel run until the dye from these tracks had reached the bottom. This allowed more of the sequence to be read, the first set of tracks were used for reading large fragments the second for the smaller fragments. After the gel had run the two plates were separated, leaving the gel attached to one plate. The gel was then fixed for 20 minutes in a 10% solution of methanol and glacial acetic acid. After fixing, a piece of blotting paper was laid on the gel, then lifted up again with the gel adhering to it. The excess blotting paper was cut away and the gel was covered with Saran wrap and trimmed. The gel was dried down at 80°C under vacuum for 45 minutes and autoradiographed with the gel surface in contact with the film.

## Chapter 3



### 3.1 Introduction

As previously described in chapter 1, in vivo deletion of a lambda phage, whose non essential EcoRI B fragment had been replaced by a 3,200 base pair palindrome (consisting of an inverted repetition of the fragment from the unique XbaI site to the second EcoRI site of lambda) gave rise to phage carrying smaller palindromes of 500-700 base pairs (Leach and Stahl, 1983). DRL110, an S7 derivative of one of these phages, has been used in this work to study the mechanism of palindrome mediated inviability. Previous to this work, the size of the palindromic remnant in DRL110 had been determined only approximately, by measuring the size of the snapback it formed in single stranded DNA from DRL107, the  $S^+$  parent of DRL110, viewed under the electron microscope (figure 3.1(i)). This gave an approximate size to the palindrome of 530 base pairs (Leach and Lindsey, 1986). The absence of any loop structure between the two inverted repeats suggested that any asymmetry at its centre was probably less than 50 base pairs. In this chapter the palindrome is characterized by sequencing to determine its exact size and the extent of any central asymmetry and also to determine the sequence surrounding the endpoints of the deletion event which lead to its formation, for comparison with in vivo deletion endpoints described by other authors.

### 3.2 Preliminary restriction analysis of DRL110

Restriction of DRL110 with EcoRI and SstI indicated that the deleted palindrome had retained the two outermost SstI sites of the large 3,200 base pair palindrome and that the two EcoRI-SstI fragments of this palindrome were the same size as each other and this fragment from normal lambda. This analysis, together with the electron micrograph snapback data and a knowledge of in vivo deletions of palindromes described by other authors, made it unlikely that these fragments had undergone any alteration during the deletion process. Therefore, it was considered necessary to sequence only the central SstI fragment. Restriction maps of the the normal lambda EcoRI B fragment, the 3,200 base pair palindrome of Leach and Stahl (1983) and the palindrome in DRL110 are shown in figure 3.1 (ii).

### 3.3 Cloning the central palindromic SstI fragment into M13 mp18

M13mp18 contains a 54 base pair polylinker containing recognition sites for 13 enzymes including SstI. Insertion of fragments into the polylinker inactivates the gene encoding  $\beta$  galactosidase and gives white instead of blue plaques on Xgal/IPTG indicator plates.

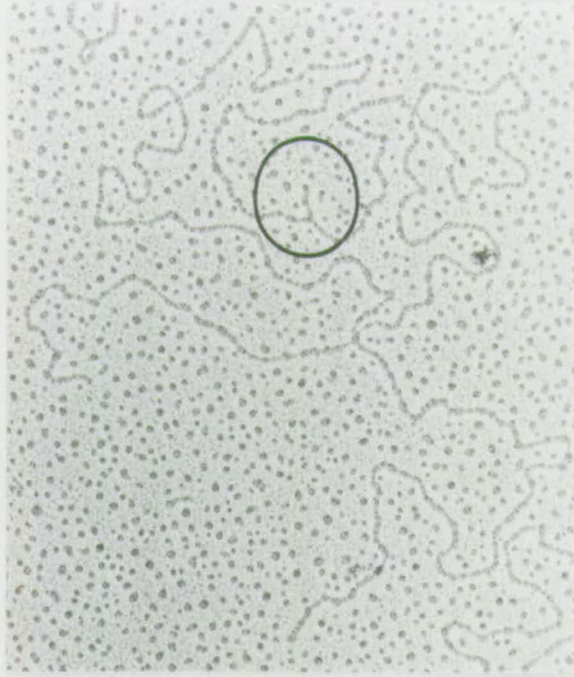
An attempt was made to clone the central SstI fragment of the 530 base pair palindrome into M13mp18 and as a control, the 1105 base pair SstI fragment of a phage JMC249, which contained the normal EcoRI B fragment of

**Figure 3.1**

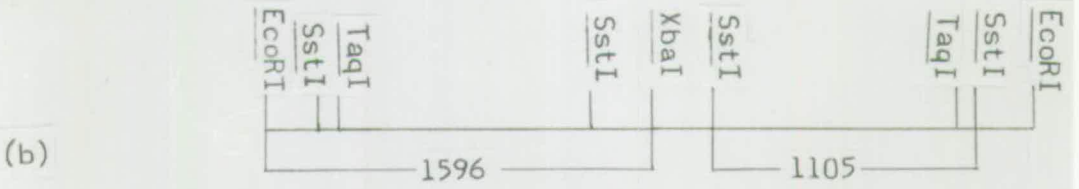
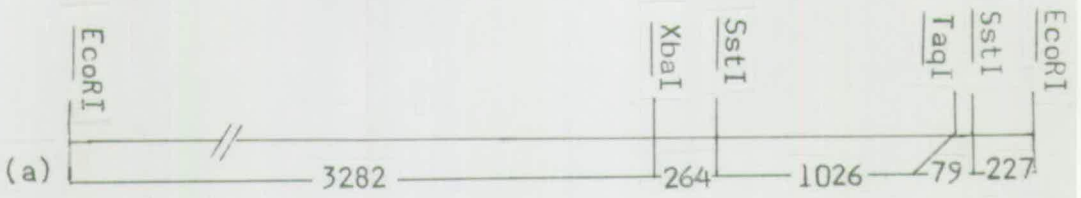
(i) Electron micrograph of a single strand of DL107 (the S<sup>+</sup> parent of DRL110) showing (within the circle) the palindrome as a double strand snap-back. Kindly provided by D. Leach.

(ii) Restriction maps of: (a) the normal lambda EcoRI B fragment, (b) the 3,200 base pair palindrome of Leach and Stahl (1983) and (c) the deleted palindrome of DRL110, showing relevant sites. Fragment sizes are given in base pairs.

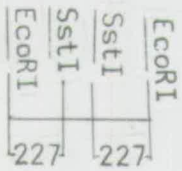
(i)



(ii)



(c)



lambda. The cloning strategy was made simple by the fact that these were the only two SstI sites in both DRL110 and JMC249. This meant that the small SstI fragments of DRL110 and JMC249 were the only fragments which could be cloned (the only other fragment bordered by SstI sites would require ligation of the cos sites and be over 40kb in length, a size far greater than M13mp18 can tolerate as an insert), therefore a fragment purification step was unnecessary. Linear DNA isolated from phage particles of DRL110 and JMC249 was digested with SstI and mixed in separate ligation reactions with SstI digested M13mp18 DNA. Following transformation of JM101 with the ligation mixes, the transformed cells were plated on Xgal/IPTG indicator plates.

The 1105 base pair SstI fragment of the control phage was cloned successfully into M13mp18, with white plaques occurring at a frequency of 25%. Several attempts were made to clone the central SstI fragment of the palindrome, in each attempt no white plaques were observed. However 10-25% of the plaques in each cloning experiment were unusual and distinctive in appearance, possessing blue and white sectors. When these 'mottled' plaques were picked and replated, they gave rise to approximately 10% white plaques and 90% blue plaques. These white and blue plaques showed no hybridisation with <sup>32</sup>P labelled lambda, nor did any of the 'mottled' or blue plaques from the original plate. The best explanation of these results is that the palindromic fragment had been cloned into M13mp18, but had rapidly deleted, totally in

cloning experiment was isolated by a large scale plasmid preparation. This plasmid could be successfully chloramphenicol amplified and isolated, but the presence of the palindrome seemed to cause a decrease in copy number of the plasmid as consistently lower yields of the DNA of this plasmid, compared to puc18, were recovered.

Restriction of this plasmid with SstI, followed by electrophoresis on a polyacrylamide gradient gel revealed the presence of an SstI fragment of approximately 110 base pairs in length, which migrated to the same size as the SstI fragment of DRL110 (figure 3.2 (iii) ), suggesting that the palindromic SstI fragment had indeed been cloned intact in puc18. The absence of a TaqI site (figure 3.2 (i)) from this palindromic fragment set the limit of any central asymmetry to less than 50 base pairs as a TaqI site lies 79 base pairs to the left of the SstI site on the lambda map (see figure 3.1 (ii) ).

### 3.6 Sequencing the palindromic SstI fragment

pJL1 was sequenced by a modification of the dideoxy chain termination method of Sanger et al. (1977) adapted for plasmid sequencing by Chen and Seeburg, (1985). The Klenow fragment of DNA polymerase I was able to replicate through the palindrome, unimpeded by any secondary structure formation. The DNA fragments appeared to run normally on the sequencing gel and there was no evidence of anomalous migration due to hairpin formation.

The sequence of one strand of the SstI fragment (the strand which was sequenced) is depicted in figure

**Figure 3.2**

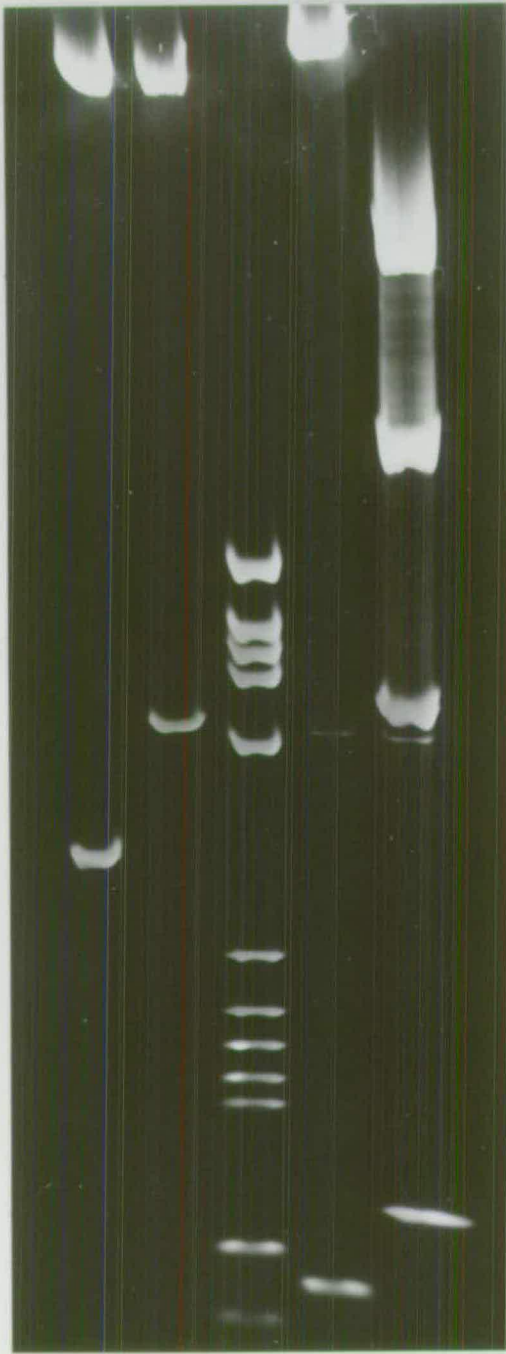
(i) pUC18 digested with PvuII (lane 1), pJL digested with PvuII, SstI and TaqI (lanes 2, 4 and 5 respectively) and pBR322 digested with HaeIII (lane 3), electrophoresed on a 3-10% polyacrylamide gradient gel. The fragments from the HaeIII digest of pBR322 were used as size markers. Fragment sizes are given in base pairs.

(ii) Restriction map of pJL1 showing relevant sites. The inverted repeats of the palindrome are depicted as arrows.

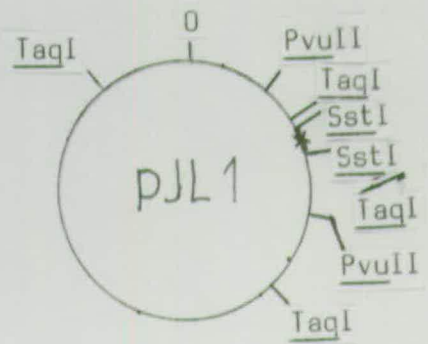
(iii) pJL1 and DRL110 DNA (lanes 2 and 3 respectively) digested with SstI and electrophoresed on a 3-10% polyacrylamide gradient gel against a HaeIII digest of pBR322 (lane 1). Note that the SstI fragment of both migrates to the same position.

(i)

1 2 3 4 5



(ii)



(iii) 1 2 3



124 / 123

110

104

89

80

~140

~110



3.3(i), drawn in a hairpin structure. From the sequence the SstI fragment was calculated to be 109 base pairs in length and shown to have a central asymmetry of 15 base pairs. The size of the palindrome in DRL110 was calculated by first adding the length of this fragment to the lengths of the two bordering EcoRI-SstI fragments. This gave a size of 563 base pairs; however the EcoRI sites themselves also contribute to the palindromic region giving another 6 base pairs not included in this calculation and by chance so does one base pair either side of the EcoRI sites. The total size of the palindrome in DRL110 was therefore calculated to be 571 base pairs

From the sequence it could be deduced that the in vivo deletion event had taken place between two 3 base pair direct repeats, within the arms of the 3,200 base pair palindrome. The sequences of the regions surrounding the deletion endpoints are given on a schematic representation of one strand of the 3,200 base pair palindrome, drawn in the hairpin configuration (figure 3.3 (iii) ). The sequence surrounding one of the direct repeats was found to possess imperfect two fold symmetry, forming a 'mini-palindrome' within the palindrome arms. This sequence is given in figure 3.3(iii) and compared to the sequences surrounding the endpoints of in vivo deletions described by other authors.

### 3.7 Discussion

The central 109 base pair SstI fragment of the palindrome present in DRL110 was cloned stably in puc18 in

**Figure 3.3**

(i) The sequence of the palindromic SstI fragment, drawn in a hairpin configuration. The SstI site is underlined.

(ii) The sequence surrounding the deletion endpoints is shown on a schematic representation of one strand of the 3,200 base pair palindrome drawn in a hairpin configuration. Deletion took place between the two direct repeats (boxed) leaving only one copy of this sequence in the deleted palindrome.

(iii)

(a) The sequence of one strand of the region surrounding one of the direct repeats is given. This region possesses two fold symmetry indicated by underlining.

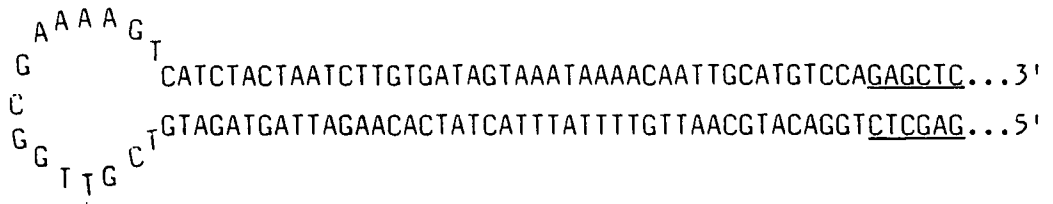
(b) Sequences of two fold symmetry surrounding the deletion endpoints of palindromes described by Yoshimura et al. (1986).

(c) Sequences of two fold symmetry surrounding the deletion endpoints of a palindrome described by Boissey and Astell (1985).

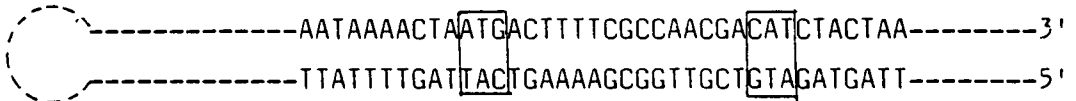
(d) Sequence of two fold symmetry surrounding one or both of the deletion endpoints of palindromes described by Collins et al. (1982).

(e) Sequence of two fold symmetry surrounding the deletion endpoint of a 'nearly precise' excision of Tn10 described by Foster et al. (1981).

(i)



(ii)



(iii)

(a) AAACTAATGACTTTT

(b) AATTGTTATCCGCTCACAATT

AGCTTATCATCGATAAGCT

(c) CAAGCGATACTCGCTTG

GACCAACCAACGCGAGTTGGTTGGTC

(d) CCATGTGACCTCCTAACATGG

(e) TAATGATTTTTATCAAAATCATT

a rec<sup>+</sup> host. The presence of the 109 base pair palindrome did not render the plasmid inviable, but it may have been associated with a reduced plasmid copy number, as consistently lower yields of pJL1 compared to puc18 were isolated from large scale plasmid preparations. This effect has been observed before with two palindromes of 146 and 147 base pairs (Warren and Green, 1985).

In contrast to its stability in puc18, the palindrome was very unstable in M13mp18. This is probably because M13 generates single stranded intermediates in its life cycle, providing increased opportunity for hairpin formation and deletion (Leach et al., 1987). Müller and Turnage (1986) showed that palindromic repeats of a BamHI linker longer than 48 base pairs could not be cloned into øX174 and that this 48 base pair sequence was rapidly deleted from the phage DNA. øX174 also generates single strand molecules in its life cycle.

The palindrome was successfully sequenced by a dideoxy chain termination method, there did not appear to be any difficulties caused by secondary structure.

From the sequence, the total size of the palindrome in DRL110 was shown to be 571 base pairs and the extent of the central asymmetry 15 base pairs.

The original in vivo deletion of the 3,200 base pair palindrome which created the 571 base pair palindrome had occurred between two short direct repeats, this is a general feature of most recA independent deletions (Farabaugh, et al., 1978; Collins et al., 1982; Yoshimura et al., 1986; DasGupta et al., 1987). The sequence surrounding

one of the direct repeats, itself possessed two fold symmetry, this has been observed previously with other palindrome deletions and these sequences are presented for comparison in figure 3.3 (iii).

In figure 3.4, a model is presented showing deletion of the 3,200 base pair palindrome to form the 571 base pair palindrome. The model postulates that deletion occurs by strand slippage during replication, similar to the mechanism proposed by DasGupta et al., 1987 and described in chapter 1. In addition it is postulated that the region of two fold symmetry may be important in promoting deletion. Separation of the nascent DNA strand from the direct repeat in this region, may be followed by the short inverted repeats surrounding it transiently forming a hairpin and temporarily 'burying' the direct repeat within this structure. This would prevent the end of the nascent DNA strand from reannealing at this site and lead to its association with the second direct repeat. This model predicts that the region of two fold symmetry need only be associated with one of the direct repeats, the 'donor' repeat, this is the case with this deletion and also several of the deletions described by Collins et al. (1982).

In summary the deletion event which created the 571 base pair palindrome of DRL110 has features in common with the deletion events described by other authors; deletion has occurred between direct repeats, one of which is in the centre of a short region of imperfect symmetry and the smaller palindrome created has an asymmetric

**Figure 3,4**

(i) The end of the nascent DNA strand end becomes separated from the template, during DNA replication.

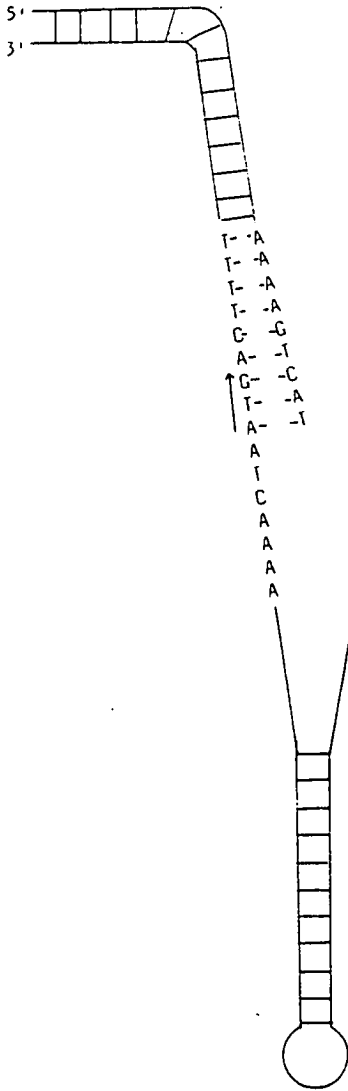
(ii) The region of two fold symmetry then base pairs to form a hairpin burying the direct repeat within it and the nascent DNA strand is forced to reanneal elsewhere (to the second direct repeat).

(iii) DNA synthesis resumes ( the structure is now drawn with the maximum possible number of base pairs formed ).

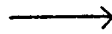
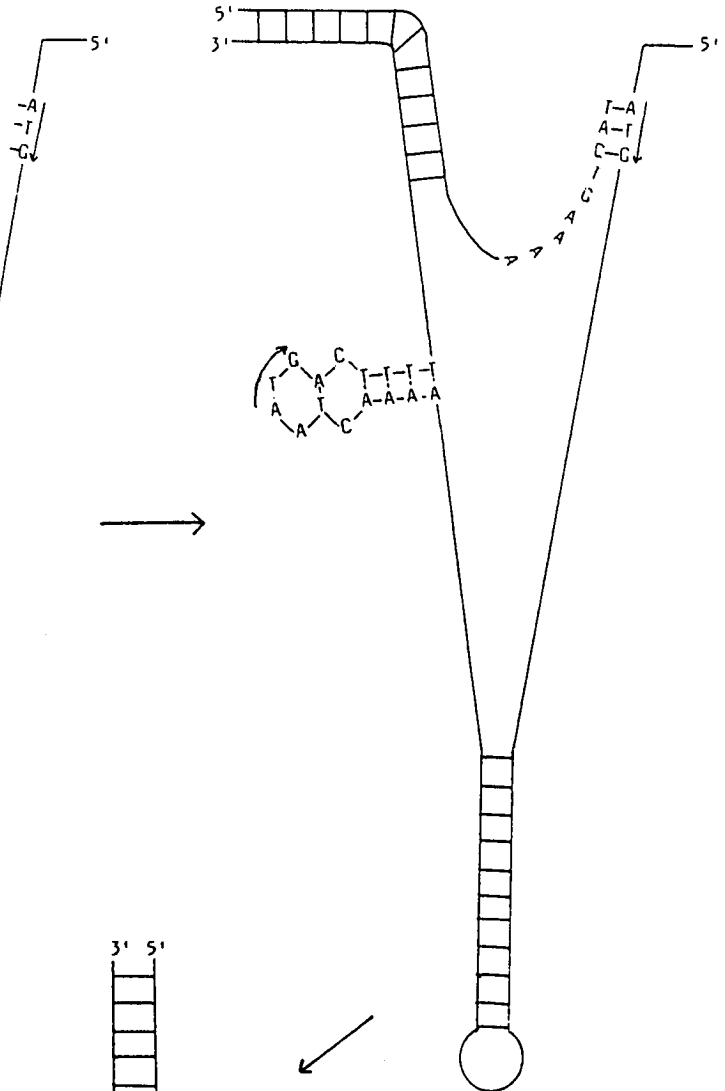
(iv) The deleted palindrome, containing only one copy of the direct repeats is formed following another round of replication.

Direct repeats are indicated by underlining with an arrow throughout.

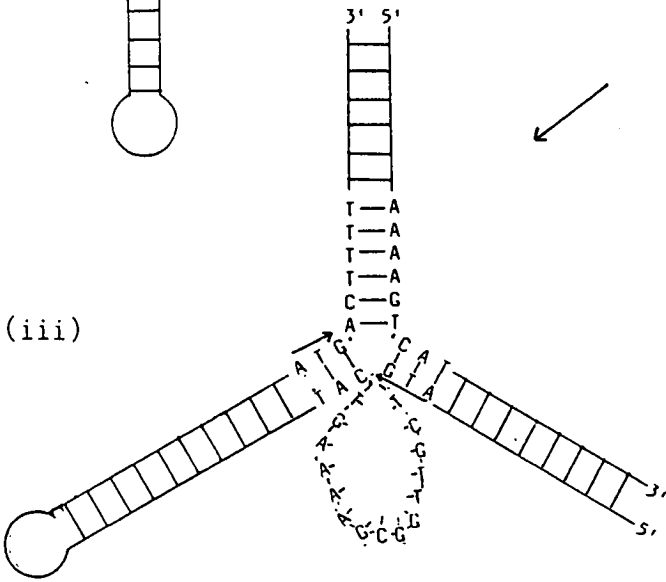
(i)



(ii)



(iii)



(iv)



centre. It is not known if all of the palindromes formed by in vivo deletion of the large 3,200 base pair palindrome have this structure. The other palindromes, although similar in size, could have occurred between other direct repeats.



## Chapter 4

#### 4.1 Introduction

The lambda phage DRL110, which has been shown to contain a 571 base pair palindrome (see chapter 3) is viable in recBC sbcB sbcC hosts, but inviable in rec<sup>+</sup> and recA backgrounds. There are many stages in the lambda life cycle (shown in figure 4.1) at which the palindrome could cause inviability. For example, it could be extruded into the cruciform conformation on supercoiling of the circular phage DNA and cleaved by nucleases as if it were a Holliday junction. Alternatively, the palindrome may interfere with replication or block packaging of the phage DNA. D. Leach (Leach and Lindsey, 1986) showed that equal amounts of the supercoiled DNA of DRL107 (the S<sup>+</sup> parent of DRL110) compared to the supercoiled DNA of a palindrome free control phage (JMC249), could be extracted following infection of a lysogen, regardless of whether the lysogen was rec<sup>+</sup> or recBC sbcB sbcC. In a lysogen the lambda prophage on the chromosome produces a repressor, so that the incoming lambda phage remains inactive in the cytoplasm following circularisation and supercoiling. From this result it appeared that the phage DNA needs to be active before an effect of the palindrome is observed. In this chapter the effect of the palindrome on lambda supercoils under conditions where the phage DNA is active is investigated.

#### 4.2 Preparation of phage stocks

Phage stocks of DRL110 (genotype red3 gam210 cI857)

**Figure 4.1** The life cycle of phage lambda (taken from G.R. Smith, 1983, also see this article for a review).

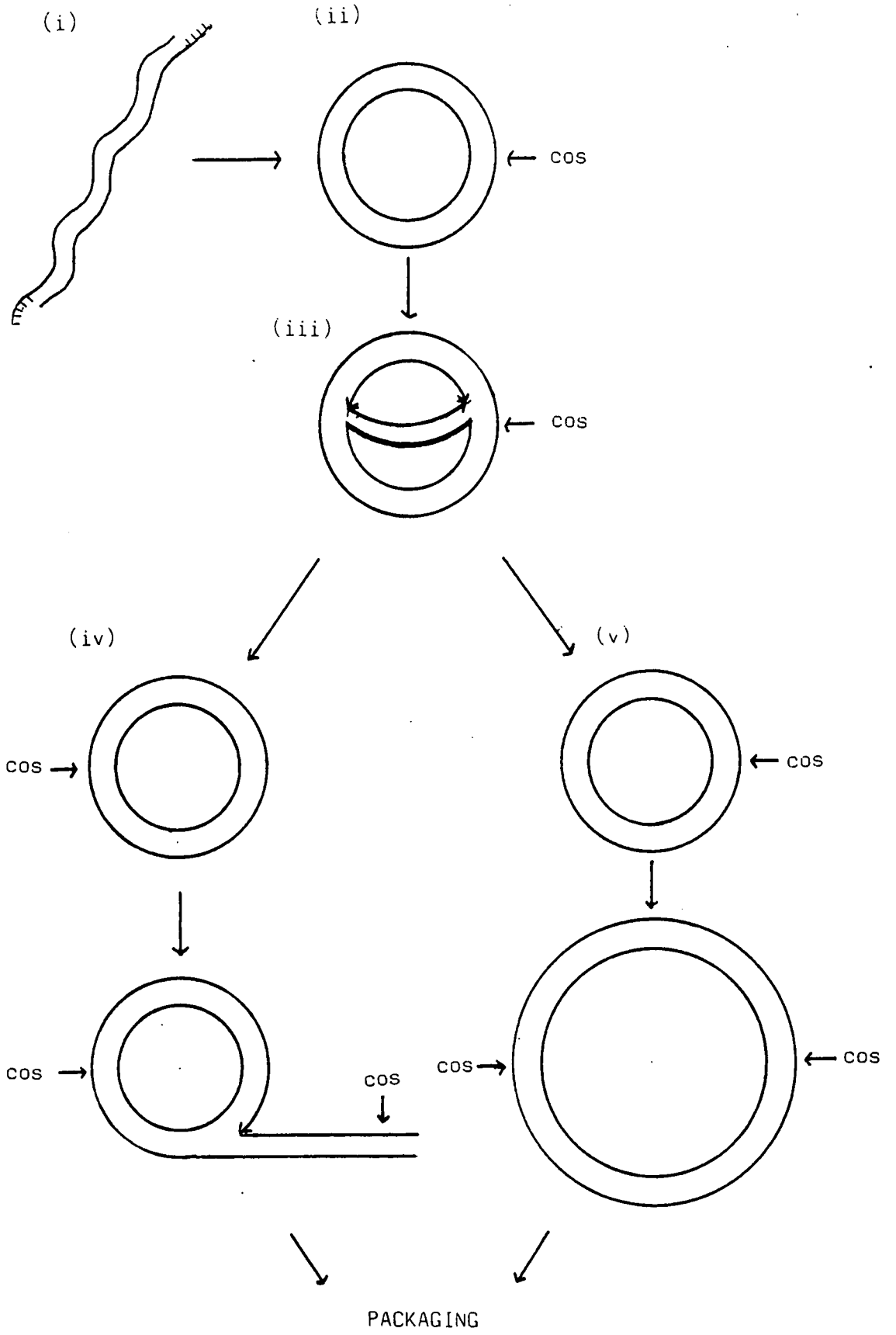
(i) Linear phage DNA is injected.

(ii) This DNA circularises by base pairing of the complementary single strand ends and is covalently joined by DNA ligase and supercoiled by DNA gyrase.

(iii) These molecules undergo a few rounds of replication in the theta mode to produce monomeric circles.

(iv) In the absence of the RecBCD nuclease ( $\text{Gam}^+$  or  $\text{RecD}^-$  infections) the molecules switch to rolling circle replication, producing long linear concatemers, which are packagable (the packaging machinery requires  $>1$  cos site per molecule).

(v) In the presence of the RecBCD nuclease ( $\text{Gam}^-$ ,  $\text{RecD}^+$  infections) replication is limited to the theta mode. Production of packagable multimers requires recombination by the lambda red pathway or the E.coli recombination pathways. In a  $\text{Red}^-$ ,  $\text{RecA}^-$ ,  $\text{Gam}^-$ ,  $\text{RecD}^+$  infection the DNA cannot be packaged as it is all monomeric.



pal571 S7) and JMC249, a palindrome free control phage (genotype spi-6 cI857 S7) were prepared by the induction of their respective lysogens, DL262 and DL190. DRL121, a phage which lacks the palindrome but is otherwise identical to DRL110, was grown as a plate lysate on JM1. Stocks of DRL110 were screened to ensure that the majority of phage retained the palindrome, by firstly selecting  $S^+$  revertants from this stock by their ability to form plaques on a recBC sbcB sbcC su<sup>-</sup> host and then picking and replating 50 such plaques on recBC sbcB sbcC su<sup>-</sup> and rec<sup>+</sup> su<sup>-</sup> hosts. Failure of these 50 plaques to replat on the rec<sup>+</sup> host was taken as evidence that they and hence the vast majority of phage in the DRL110 phage stock retained the palindrome. Because of the presence of the S7 mutation in DRL110 its plating ability on rec<sup>+</sup> hosts cannot be tested directly as suppression of the S7 mutation would also result in suppression of the gam210 mutation and permit plating on rec<sup>+</sup> su<sup>+</sup> hosts. S7 phages were used in the infection experiments described below to prevent phage induced lysis of the cells.

#### 4.3 Infection of non lysogenic rec<sup>+</sup> and recBC sbcB sbcC strains with DRL110 and JMC249

JC9387 (recBC sbcB sbcC) and JC9937 (rec<sup>+</sup>) were infected with a mixture of DRL110 and JMC249 at a multiplicity of infection of 2 for each phage. After 10 minutes of exponential growth (a 10 minute infection time was used to minimise the amount of rolling circle replication in the recBC sbcB sbcC strain and to prevent

the DNA packaging into phage heads), the cells were lysed and supercoiled DNA prepared on a CsCl/EtBr gradient. Since the two DNA molecules have indistinguishable mobilities on agarose gels, the supercoiled fraction was subjected to digestion with the restriction enzyme EcoRI. As DRL110 and JMC249 carry deletions in different parts of their genomes, fragments specific to each molecule are generated by cleavage (see figure 4.2). The DNA was electrophoresed on an agarose gel and in order to increase the sensitivity of the experiment, the restriction fragments were transferred to a nitrocellulose filter and hybridised to  $^{32}\text{P}$  labelled lambda DNA.

As can be seen from figure 4.2 (ii), the ratio of supercoiled DNA of DR110 to JMC249 extracted from rec<sup>+</sup> cells is lower than that extracted from recBC sbcB sbcC cells. The yields of the two DNAs were quantified by densitometry. The ratio of band P (unique to DRL110) to band S<sub>1</sub> (unique to JMC249) was calculated to be 0.18 for the DNA extracted from the rec<sup>+</sup> strain and 0.55 for the DNA extracted from the recBC sbcB sbcC strain. The relative recovery of palindrome containing DNA from the rec<sup>+</sup> strain compared to the recBC sbcB sbcC strain was calculated as:

$$R_{(P/S)} = (P/S_1 \text{ rec^+}) / (P/S_1 \text{ recBC sbcB sbcC})$$

This gave a value of  $R_{(P/S)} = 0.33$ . As an internal control the ratios of band S<sub>1</sub> to S<sub>2</sub> in tracks 1 and 2 were calculated. S<sub>1</sub> and S<sub>2</sub> are both fragments unique to JMC249, so the ratio of the two bands in each lane should be the same. The ratio of S<sub>1</sub> to S<sub>2</sub> was calculated to be 9.4 for

**Figure 4.2**

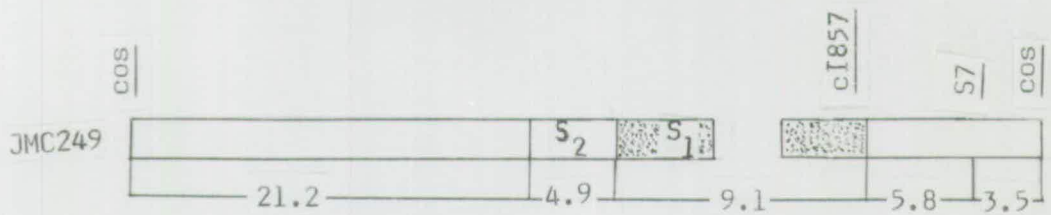
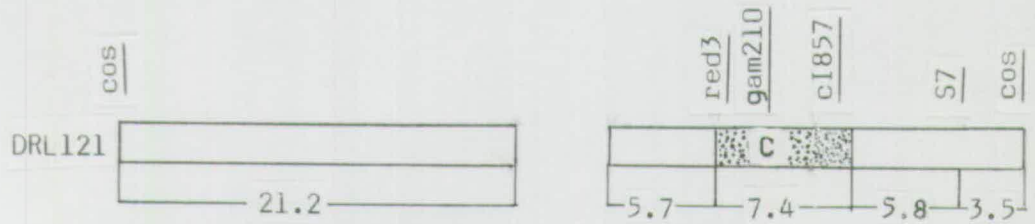
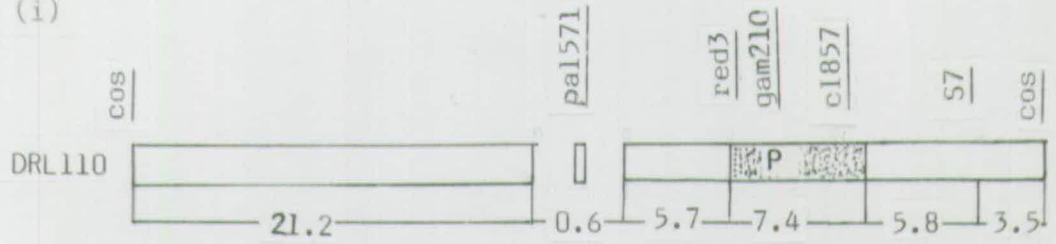
(i) EcoRI restriction maps of the phages used showing DNA structure and genotype. Deletions ( $\Delta B$  in DRL110 and DRL121,  $\Delta spi6$  in JMC249) are shown as interruptions in the genome. Fragments P, C and  $S_1$  (shaded) were used to quantitate the recovery of supercoiled molecules,  $S_2$  was used as an internal control (see text).

(ii) DNA recovered from rec<sup>+</sup> and recBC sbcB sbcC strains following infection with DRL110 and JMC249. EcoRI digests of: supercoiled DNA of DRL110 + JMC249 recovered from the rec<sup>+</sup> infection (lane 1) and the recBC sbcB sbcC infection (lane 2) and linear DNA from DRL110 (lane 3) and JMC249 (lane 4) extracted from phage particles. The presence of bands generated by denaturation of the cohesive ends (by heating to 70°C for 10 minutes following digestion) in the linear DNA tracks and the absence of these bands from the tracks with circular DNA was taken as evidence that contamination of the supercoils with linear DNA from phage particles which have not injected their DNA was undetectable.

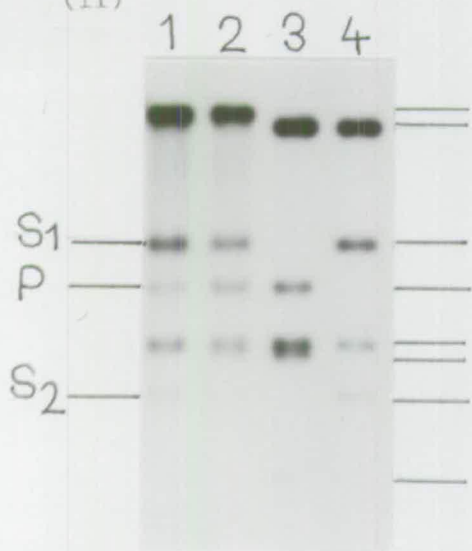
(iii) DNA recovered from rec<sup>+</sup> and recBC sbcB sbcC strains following infection with DRL121 and JMC249. EcoRI digests of: linear DNA from DRL110 (lane 1) and JMC249 (lane 2) extracted from phage particles and supercoiled DNA of DRL121 + JMC249 recovered from the rec<sup>+</sup> infection (lane 3) and the recBC sbcB sbcC infection (lane 4). No contamination of supercoiled DNA with linear phage DNA was detected.

Fragments sizes are given in kilobase pairs throughout.

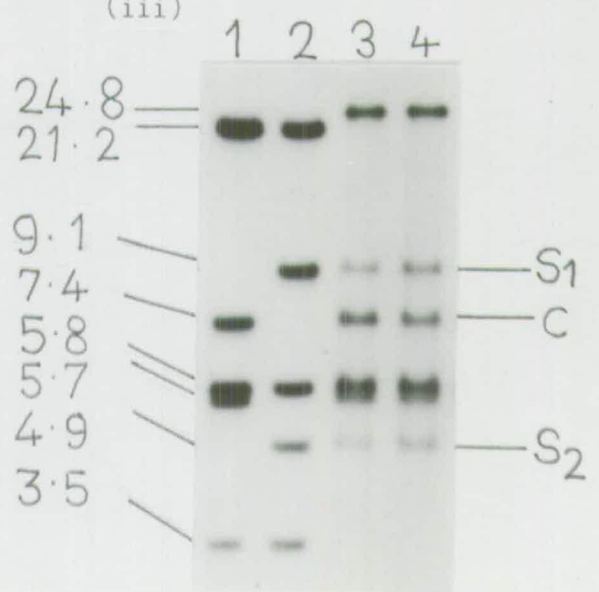
(i)



(ii)



(iii)





the DNA extracted from the rec<sup>+</sup> host and 10.8 for the DNA extracted from the recBC sbcB sbcC host. The ratio of these two values I was calculated as:

$$I(S_1/S_2) = (S_1/S_2 \text{ rec<sup>+</sup>}) / (S_1/S_2 \text{ recBC sbcB sbcC})$$

This gave a value of  $I = 0.87$ , which is approaching the expected ratio of 1. This ratio provides a measure of the interlane variance, due to such effects as differences in transfer efficiency and sets a limit on the significance of R. If R had approached the value of I then this would have meant that there was no significant difference in the recovery of palindrome containing supercoils from rec<sup>+</sup> and recBC sbcB sbcC hosts.

#### 4.4 Infection of rec<sup>+</sup> and recBC sbcB sbcC strains with DRL121 and JMC249

To confirm that this loss of supercoiled DNA observed in a rec<sup>+</sup> strain was due to the presence of the palindrome and not to some other difference between the two DNAs a control experiment was performed in an identical manner to the above experiment except that DRL110 was replaced with DRL121, a phage which lacks the palindrome but is otherwise identical to DRL110 (see figure 4.2 (i) ).

The results of this experiment are shown in figure 4.2 (iii) where it can be seen that the supercoiled DRL121 DNA is efficiently recovered from both rec<sup>+</sup> and recBC sbcB sbcC hosts. The ratio of band C (unique to DRL121) to band S<sub>1</sub> (unique to JMC249) was calculated to be 2.9 for the DNA extracted from the rec<sup>+</sup> strain and 1.5 for the DNA

extracted from the recBC sbcB sbcC strain. The relative recovery of DRL121 was calculated as:

$$R_{(C/S)} = (C/S_1 \text{ rec}^+) / (C/S_1 \text{ recBC sbcB sbcC})$$

This gave a value of  $R=1.9$ . The corresponding I ratio was 0.99 therefore it is unlikely that this R value is due to transfer differences. This result shows that supercoiled DRL121 DNA is recovered slightly better than supercoiled JMC249 DNA from the rec<sup>+</sup> host. This is probably due to the difference in genotypes of the two phages. If so, this effect will partially mask the loss of palindrome containing DNA molecules. Using the two values  $R_{(P/S)}$  and  $R_{(C/S)}$  the value  $R_{(P/C)}$  can be calculated as:

$$R_{(P/C)} = R_{(P/S)} / R_{(C/S)}$$

This gives a value of  $R_{(P/C)} = 0.17$ . This is a truer estimation of the depression in the level of supercoils due to the palindrome, as it compares DRL110 and DRL121 which differ only by the presence of the palindrome. This value of  $R_{(P/C)}$  is confirmed below.

#### 4.5 Infection of rec<sup>+</sup> and recBC sbcB sbcC strains with DRL110 and DRL121

To confirm the above experiments and the lysogen experiment of D. Leach (Leach and Lindsey), 10 minute infections of JC9387 ( recBC sbcB sbcC ), JC9937 (rec<sup>+</sup>) and DL187 (rec<sup>+</sup>, lambda lysogen) were carried out using DRL121 and DRL110. DRL121 is the ideal control phage for DRL110. Nevertheless these phages are not an easy combination to use as they differ by only 563 base pairs (the size of the palindromic EcoRI fragment), which means

that it is difficult to find an enzyme which will give restriction fragments unique to each phage, sufficiently far apart from one another and other fragments to be traceable by densitometry for quantification. The enzyme chosen was AvaI, this gives a fragment of 2.6kb from DRL110, which can be traced by densitometry and a fragment of 2.0kb from DRL121 which can be seen on the autoradiograph but is too similar in size to a fragment of 1.9kb for densitometer tracing.

As can be seen from figure 4.3 (ii), the palindrome containing supercoils are recovered well from the recBC sbcB sbcC strain and the rec<sup>+</sup> lysogen, but in reduced amounts from the rec<sup>+</sup> non lysogen. The ratio of DRL110 to DRL121 supercoils was calculated as follows. The 2.6kb fragment (unique to DRL110, labelled P in figure 4.3) was quantified by densitometry as was the 3.7kb fragment (derived from both phages, labelled PC in figure 4.3). The contribution that DRL110 made to fragment PC was estimated as:

$$P_{(PC)} = P \times (3.7/2.6)$$

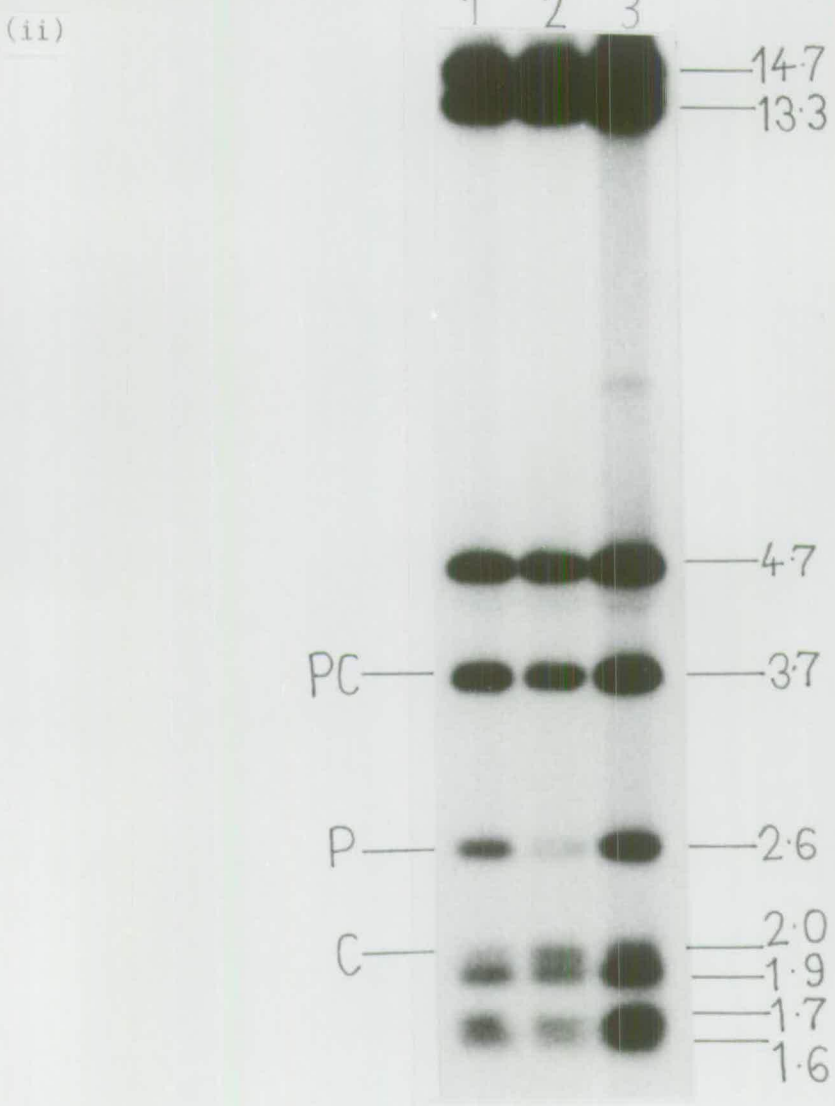
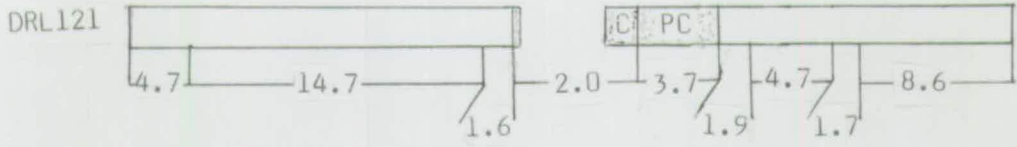
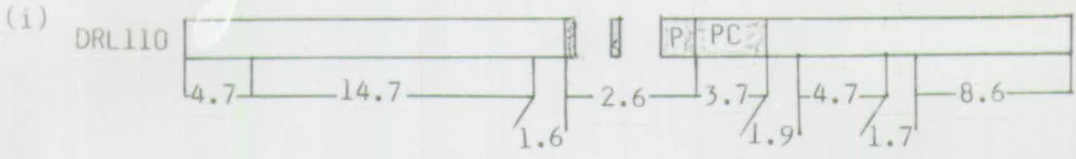
to compensate for the differences in size of the two fragments. This was then subtracted from the value of PC to give C<sub>(PC)</sub>, the contribution of DRL121 to this fragment. The ratio of P<sub>(PC)</sub> to C<sub>(PC)</sub> was then calculated and found to be 0.71 for the DNA extracted from the recBC sbcB sbcC host, 0.13 for the DNA extracted from the rec<sup>+</sup> host and 1.1 for the DNA extracted from the lysogen. The recovery of DL110 DNA from the rec<sup>+</sup> infection relative to the recBC sbcB sbcC infection was calculated as:

**Figure 4.3**

(i) AvaI restriction maps of DRL110 and DRL121. Deletions are shown as interruptions in the genome. Fragments P and C (shaded) are unique to DRL110 and DRL121 respectively, fragments P and PC (also shaded) were used to quantitate the recovery of supercoiled molecules.

(ii) DNA recovered from rec<sup>+</sup> and recBC sbcB sbcC non lysogens and a rec<sup>+</sup> lysogen following infection with DRL110 and DRL121. AvaI digests of supercoiled DNA of DRL110 + DRL121 recovered from the recBC sbcB sbcC non lysogen (lane 1), the rec<sup>+</sup> non lysogen (lane 2) and lysogen (lane 3).

Fragment sizes are given in kilobase pairs throughout.



$$R_{(P/C)} = (P/C \text{ rec^+}) / (P/C \text{ recBC sbcB sbcC})$$

this gave a value of  $R_{(P/C)} = 0.18$ . This is in very close agreement with the value of  $R_{(P/C)}$  of 0.17 obtained from the two previous experiments. The recovery of DRL110 DNA relative to the lysogen infection was 0.12 for the DNA extracted from the rec<sup>+</sup> strain and 0.65 for the DNA extracted from the recBC sbcB sbcC strain, suggesting that the palindrome might cause a slight decrease in the level of actively replicating supercoils even in the recBC sbcB sbcC strain.

#### 4.6 Infection of a recA strain with DRL110 and JMC249

To observe the effects of longer periods of activity it was necessary to block DNA packaging. This was done by infecting a recA host (W3101 RecA<sup>-</sup>). In recA strains red gam phage cannot package their DNA as it is all monomeric (see figure 4.1). DNA was extracted after infection of a recA strain with equal numbers of JMC249 and DRL110 (m.o.i. of 2 for each phage) following 10 and 60 minutes of exponential growth. As for previous experiments supercoils were prepared on a CsCl/EtBr gradient, subjected to digestion with EcoRI, electrophoresed on an agarose gel, transferred to nitrocellulose and hybridised to <sup>32</sup>P labelled lambda DNA.

As can be seen from figure 4.4(ii), the loss of palindrome containing supercoils is greater at 60 minutes than at 10, demonstrating that the decrease is a progressive one rather than due to a single event. The relative recovery of palindrome containing supercoils,

**Figure 4.4**

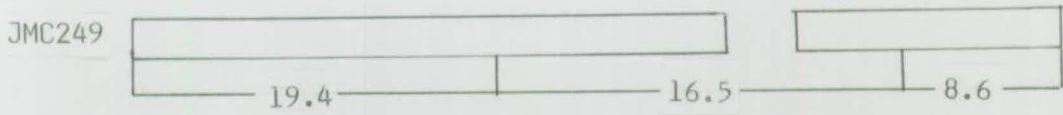
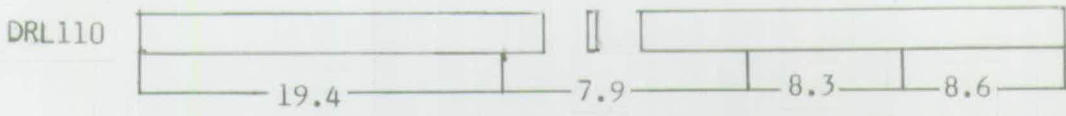
(i) SmaI restriction maps of DRL110 and JMC249. Deletions are shown as interruptions in the genome.

(ii) DNA recovered from recA infections following 10 and 60 minutes of growth. EcoRI digests of supercoiled DNA of DRL110 + JMC249 recovered after 10 (lane 1) and 60 (lane 2) minutes and linear DNA of JMC249 (lane 3) and DRL110 (lane 4) extracted from phage particles. No contamination of the supercoiled fraction with linear phage DNA is detectable (fragments formed by denaturation of the cos sites are absent).

(iii) SmaI digest of linear DNA of JMC249 (lane 1) and DRL110 (lane 2) extracted from phage particles and supercoiled DNA of DRL110 + JMC249 recovered after 60 minutes (lane 3).

Fragment sizes are given in kilobase pairs throughout.

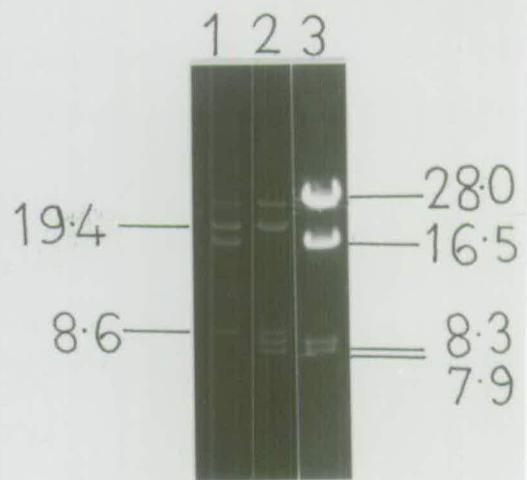
(i)



(ii)



(iii)





R(p/S) compared to the simultaneous infection of DL187 (a rec<sup>+</sup> lysogen, not shown in figure 4.4 (ii) ) was calculated and found to be 0.14 at 10 minutes and 0.04 at 60 minutes. The corresponding I values were 0.75 and 0.81 respectively.

In order to determine whether the DNA from DRL110, present after 60 minutes of replication still retained the palindrome, the DNA was digested with SmaI and electrophoresed on an agarose gel (there was sufficient supercoiled DNA extracted from the 60 minute infection to be able to see directly on the agarose gel, without the need for transfer and hybridisation to radioactive DNA). SmaI digestion of the DNA gives two bands unique to DRL110 of similar size (8.3kb and 7.9kb), the latter of which contains the palindrome (see figure 4.4 (i) ). If the palindrome had deleted from the supercoils recovered in this experiment, the smaller band would be reduced to 7.4kb in size. This has not occurred in the vast majority of molecules, as the two original bands are present in equal amounts and no additional bands can be seen (figure 4.4 (iii) ).

#### 4.7 Discussion

An effect of the palindrome on actively replicating lambda supercoils has been demonstrated. Previously it had been shown by D. Leach (Leach and Lindsey, 1986) that equal numbers of supercoils of a palindrome containing phage compared to a palindrome free control could be extracted following infection of both rec<sup>+</sup> and recBC sbcB

sbcC lysogens, where the DNA is inactive. In contrast the palindrome containing supercoils were poorly recovered following infection of non lysogenic rec<sup>+</sup> and recA cells but efficiently recovered following infection of recBC sbcB sbcC cells. The palindrome appears to cause a progressive decrease in the levels of lambda supercoils, the products of theta replication, in strains where it confers inviability.

These results rule out the hypothesis that the palindrome causes inviability solely by interfering with the packaging of the phage DNA.

The requirement of the DNA to be active can be explained in several ways. The palindrome may interfere with an aspect of active DNA, such as transcription or replication. A sequence capable of forming Z DNA has been shown to cause blockage of transcription of supercoiled DNA in vitro (Peck and Wang, 1985). However, replication was considered the most likely of these two alternatives. Poor transcription of palindrome containing DNA is unlikely to be the cause of DNA loss, as complementation will occur from JMC249, which will be present in more than 85% of cells infected with DRL110 at the multiplicities used. Replication is also strongly implicated in the results of Shurvinton et al. (1987), who found that phage carrying large palindromes could be recovered well from phage particles carrying unreplicated chromosomes but poorly from phage carrying replicated chromosomes, following infection of a rec<sup>+</sup> host.

Alternatively, instead of direct interference of

the palindrome with replication, the strand separation involved in replication may catalyse extrusion of the palindrome into a cruciform conformation, which may then be cleaved by nucleases. Another possibility is that replication creates an entry site for an enzyme which cleaves cruciforms.

Supercoil loss appears to be almost totally alleviated in recBC sbcB sbcC strains. It is now known that it is the sbcC mutation which is important (A. Chalker, personal communication). The sbcC gene product may be a nuclease able to cleave cruciforms or hairpins, an unwinding protein capable of catalysing cruciform extrusion, or a protein involved in some aspect of replication.

The mechanism of inviability is further investigated in chapter 5.

## Chapter 5

## 5.1 Introduction

It has been shown (see chapter 4) that the presence of a palindrome in a lambda phage decreases the level of phage supercoils, compared to the levels of a palindrome free control. This reduction occurs in strains where the palindrome containing phage is inviable providing the DNA is active (not repressed). Two very general explanations of this decrease are possible; either the palindrome containing DNA is destroyed, perhaps due to mistaken recognition of the cruciform for a Holliday junction, or the palindrome replicates more slowly than the palindrome free control, perhaps due to the formation of hairpins which impede DNA synthesis. An attempt to distinguish between these two hypotheses was made using the following strategy.

Phage stocks of DRL110 (the palindrome containing phage) and JMC249 (the palindrome free control), whose DNA was modified against cleavage with a restriction enzyme (MboI or EcoRI depending on the experiment) were used to infect a host not possessing the appropriate methylase (dam or EcoRI). Replication of the phage DNA generated hemimethylated and fully unmethylated molecules. Molecules carrying two DNA strands newly formed by replication (fully unmethylated) could be distinguished from molecules retaining one input strand (hemimethylated), as the latter were resistant to cleavage. After the first round of replication, the number of hemimethylated DNA molecules of the palindrome containing phage should remain constant

unless destruction occurs (see table 5.1). The levels of hemimethylated palindrome containing DRL110 DNA relative to hemimethylated palindrome free JMC249 DNA could therefore be monitored to see if a decrease of DRL110 DNA occurred. Such a decrease would have indicated destruction of the palindrome containing DNA.

## 5.2 Preparation of phage stocks

### (i) Preparation of dam modified phage stocks

The dam gene of E.coli codes for an enzyme which methylates the N-6 position of deoxyadenosine residues in the sequence GATC (Marinus and Morris, 1973). The DNA of lambda phage grown in normal E.coli strains is incompletely methylated, so a strain carrying pTP166, a multicopy plasmid carrying the dam gene (Marinus et al., 1984) was used to prepare phage stocks. The dam gene of this plasmid is transcribed from a tac promoter and transcription from this promoter is repressed by the lacI product. Induction with IPTG alleviates this repression and results in overexpression of dam.

Phage stocks of DRL110 and JMC249 were grown by one step liquid lysis in JL28, a recBC sbcA supE strain carrying pTP166 (the supE mutation suppresses the gam210 mutation of DRL110, but not the S7 mutation, the liquid lysis method used was therefore that described in chapter 2 for phages with an unsuppressed S mutation). A recBC sbcA supE strain was chosen as opposed to a recBC sbcB sbcC strain, for growth of DRL110 as ColE1 derivative

plasmids such as pTP166, are highly unstable in recBC sbcB sbcC strains (Basset and Kushner, 1984; Cohen and Clark, 1985). The palindrome containing phage stock was screened to ensure the phage retained the palindrome, as described in section 4.2. No phage which had deleted the palindrome were found in the 50 phages tested and so it was assumed that any deletants would not be present in sufficient numbers to influence the results.

Phage stocks grown by this method were low titre ( approximately  $5 \times 10^9$ /ml ) despite the fact that 2 litres of bacterial culture (at  $2 \times 10^8$  cells/ml) infected at a multiplicity of 5 were used to produce only 50ml of phage stock. This meant that there were insufficient phage to prepare linear DNA from by the method described in section 2.2.19 in order to determine whether the DNA was fully methylated. Instead the dam methylated JMC249 and DRL110 phage stocks were used to co-infect a dam<sup>-</sup> lysogen, followed by extraction of the DNA and preparation of the supercoils on a CsCl/EtBr gradient. No replication of the phage DNA should occur on infection of a lysogen and so the methylation status of the supercoils extracted from the lysogen should reflect the original methylation status of the two phage stocks. The supercoiled DNA was digested with EcoRI to generate fragments specific to each phage (see section 4.2 for EcoRI restriction maps of the two phage). One third volume of DNA was then removed and the other two thirds were further digested with MboI. Finally one half of this EcoRI + MboI digested DNA was further digested with DpnI. These three digests were

electrophoresed on a agarose gel, followed by transfer to a nitrocellulose filter and hybridisation to radioactively labelled phage lambda DNA. MboI cuts the sequence GATC when it is unmethylated, whereas DpnI cuts the same sequence when it is fully methylated, however neither enzyme will cleave a hemimethylated sequence (Vovis and Lacks, 1977). MboI and DpnI cut DRL110 and JMC249 DNA at more than 80 sites, therefore the cleavage products are very small and do not interfere with the EcoRI digestion fragments. This method of sequential digestion, with removal of a fraction of DNA after each digestion allows the ratio of the two phage DNAs in the total DNA (fully methylated + hemimethylated + unmethylated), the methylated DNA (fully + hemimethylated) and the hemimethylated DNA only, to be calculated by densitometer tracing of the EcoRI fragments.

From figure 5.1 it can be seen that the majority of the phage DNA is methylated (resistant to MboI digestion) however some MboI cleavage products can be seen indicating that not all the phage have DNA which is methylated at every site. This is presumably because of plasmid loss from the recBC sbca strain, as ColE1 derivative plasmids show some instability in such strains (Summers and Sherratt, 1984). The ratio of DRL110 to JMC249 was calculated using bands P and S, for the total DNA and the fully modified DNA. This gave a ratio of P/S of 0.97 for the total DNA and 0.93 for the fully modified DNA, showing that there was not a significant difference in the methylation status of each phage stock. It can also



**Figure 5.1**

DRL110 + JMC249 supercoiled DNA recovered following infection of a dam<sup>-</sup> rec<sup>+</sup> lysogen with dam methylated phage, digested sequentially with EcoRI (lane 1) + MboI (lane 2) + DpnI (lane 3). The EcoRI digest fragments in lane 1 therefore consist of total DNA (fully methylated + hemimethylated + unmethylated), in lane 2 of fully methylated + hemimethylated DNA and in lane 3, hemimethylated DNA only. Bands P and S were used to compare the yields of the two phage DNAs.

The pBR322 band in lane 1, which could be seen clearly on the agarose gel and faintly on the autoradiograph consists of unmethylated pBR322 DNA (added to the DNA extracted from JL33, before isolation of the supercoils on a CsCl/EtBr gradient) and fully methylated DNA (added just before EcoRI digestion). EcoRI cuts pBR322 once. The absence of the pBR322 band in lane 3 was taken as evidence that MboI and DpnI had digested properly, so that the hemimethylated EcoRI lambda fragments in lane 3 were unlikely to be contaminated with any undigested fully or unmethylated DNA. This method of confirming digestion can be seen more clearly in figure 5.2 (ii).

Fragment sizes are given in kilobase pairs.



be seen from figure 5.1 that there is virtually no hemimethylated DNA present, this extremely small amount of hemimethylated DNA initially present in the two phage stocks is unlikely to influence the results.

#### 5.2(ii) Preparation of EcoRI modified phage stocks

JMC249 was plated on JL5 a recB sbcA supF strain carrying pMB1, a plasmid expressing the EcoRI restriction and modification genes (Yoshimori et al., 1972). This phage gave a reduced plating efficiency ( $10^{-5}$  to  $10^{-6}$ ) on this strain, compared to the same strain not carrying the plasmid (JM1), due to destruction of the phage DNA by the restriction enzyme. A plaque which had escaped restriction and therefore contained phage whose DNA was modified (phage whose DNA is resistant to cleavage will occur at extremely low frequencies as this would require mutation of all 4 EcoRI recognition sites) was picked and used to produce a plate lysate on JL5. To test whether this phage stock was fully modified it was plated on JL5 (JM1/pMB1) and JM1. The phage stock plated with the same efficiency on both strains suggesting that the phage DNA was probably fully modified. This modification status was confirmed by restriction of the DNA following infection of a lysogen (see section 5.5 and figure 5.5).

This method could not be used for growth of DRL110, as plate stocks of DRL110 grown on recB sbcA strains contain a high proportion of palindrome free deletants. Instead DL262, a recBC sbcB sbcC strain lysogenic for DRL110 was transformed with pMB1. A phage

stock was then produced by lysogenic induction of this strain. This phage stock plated with an efficiency of 25% on JL5 (JM1/pMB1) compared to JM1 suggesting that it was not fully modified. This was probably due to plasmid loss from DL262, because as mentioned previously ColE1 derivatives are extremely unstable in recBC sbcB sbcC strains. These phage were then used to infect JL5, for production of a phage stock by one step liquid lysis (the supF mutation of JL5 suppresses the S7 mutation of DRL110, the liquid lysis method used was therefore that described in chapter 2 for phage with a suppressed S mutation). The resulting phage stock was fully modified as determined by its plating efficiency on JL5 compared to JM1 and analysis of its DNA following infection of a lysogen (figure 5.5). The phage had only undergone one or two rounds in JL5, so the vast majority of phages retained the palindrome, shown by the absence of palindrome free phages in 50 phages tested in the manner described in section 4.2.

### 5.3 Infection of a $dam^- rec^+$ strain with $dam$ modified DRL110 and JMC249

A  $dam^- rec^+$  strain JL30 was infected with equal numbers of  $dam$  methylated DRL110 and JMC249 at a multiplicity of 2 for each phage. The DNA was extracted and the supercoils prepared on a CsCl/EtBr gradient following 7 and 15 minutes of exponential growth (15 minutes was judged to be the longest permissible infection time as at later times the DNA would start to be packaged into phage heads). A 7 minute infection of a  $dam^- rec^+$

lambda lysogen (JL33) was carried out in parallel. Phage supercoils were digested with EcoR1 to generate fragments specific to each phage. Portions of phage supercoils from the non lysogen infection were further digested with MboI or MboI + DpnI in the manner described in section 5.2 (i). The digested samples were electrophoresed on an agarose gel, transferred to nitrocellulose and hybridised to  $^{32}\text{P}$  labelled lambda DNA. As described previously in chapter 4, there is no loss of palindrome containing supercoils following infection of a lysogen, so the ratio of DRL110 DNA to JMC249 DNA in the supercoils extracted from the lysogen could be taken as the initial ratio of the two phage DNAs. A comparison with this ratio determined whether DRL110 supercoil loss had occurred. As can be seen from figure 5.2, the levels of the total palindrome containing supercoils (fully methylated + hemimethylated + unmethylated) had progressively decreased with time, compared to the lysogen infection. The ratio of band P (unique to DRL110) to band  $S_1$  (unique to JMC249) was found to be 0.67 for the lysogen, 0.41 for the 7 minute infection and 0.12 for the 15 minute infection. The relative recovery of palindrome containing DNA was calculated as:

$$R_T = (P/S_1 \text{ non lysogen}) / (P/S_1 \text{ lysogen})$$

This gave a value of  $R_T = 0.6$  at 7 minutes and  $R_T = 0.19$  at 15 minutes. The ratio of  $S_1$  to  $S_2$  (calculated as an internal check, see chapter 4 for details) could not be determined in these tracks as this band was obscured by anomalous migration of the unmethylated pBR322 DNA (see

**Figure 5.2**

(i) JMC249 + DRL110 supercoiled DNA recovered following infection of a rec<sup>+</sup> dam<sup>-</sup> lysogen and non lysogen. DNA recovered from: the lysogen digested with EcoRI (lane 1); the non lysogen after 7 minutes of growth digested sequentially with EcoRI (lane 2) + MboI (lane 3) + DpnI (lane 4) and the non lysogen after 15 minutes of growth digested sequentially with EcoRI (lane 5) + MboI (lane 6) + DpnI (lane 7). EcoRI restriction fragments in lanes 1, 2 and 5 therefore consist of total DNA (fully methylated + hemimethylated + unmethylated), in lanes 3 and 6 of fully and hemimethylated DNA and in lanes 5 and 7 of hemimethylated DNA only. Bands P and S<sub>1</sub> were used to quantify yields of the two phage DNAs. Twice as much DNA was loaded in lanes 3, 4 and 6, 7 compared to lanes 2 and 5 respectively. Fragment sizes are given in kilobase pairs.

(ii) Section of agarose gel before transfer to nitrocellulose showing pBR322 band. The pBR322 band in tracks 1, 2 and 5 consists of approximately equal amounts of dam fully methylated and unmethylated DNA. The absence of the pBR322 band from lanes 4 and 7 is taken as evidence that MboI and DpnI have fully digested the DNA and therefore there is no contamination of hemimethylated lambda EcoRI fragments with unmethylated or fully methylated DNA.

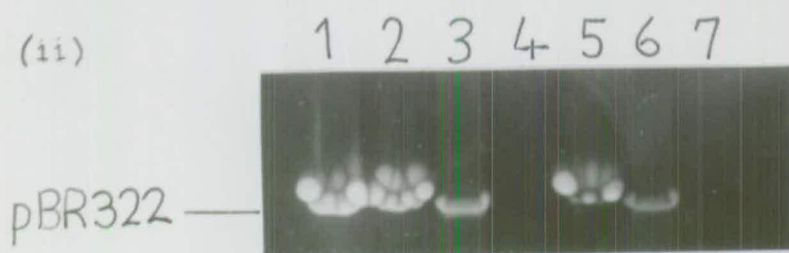
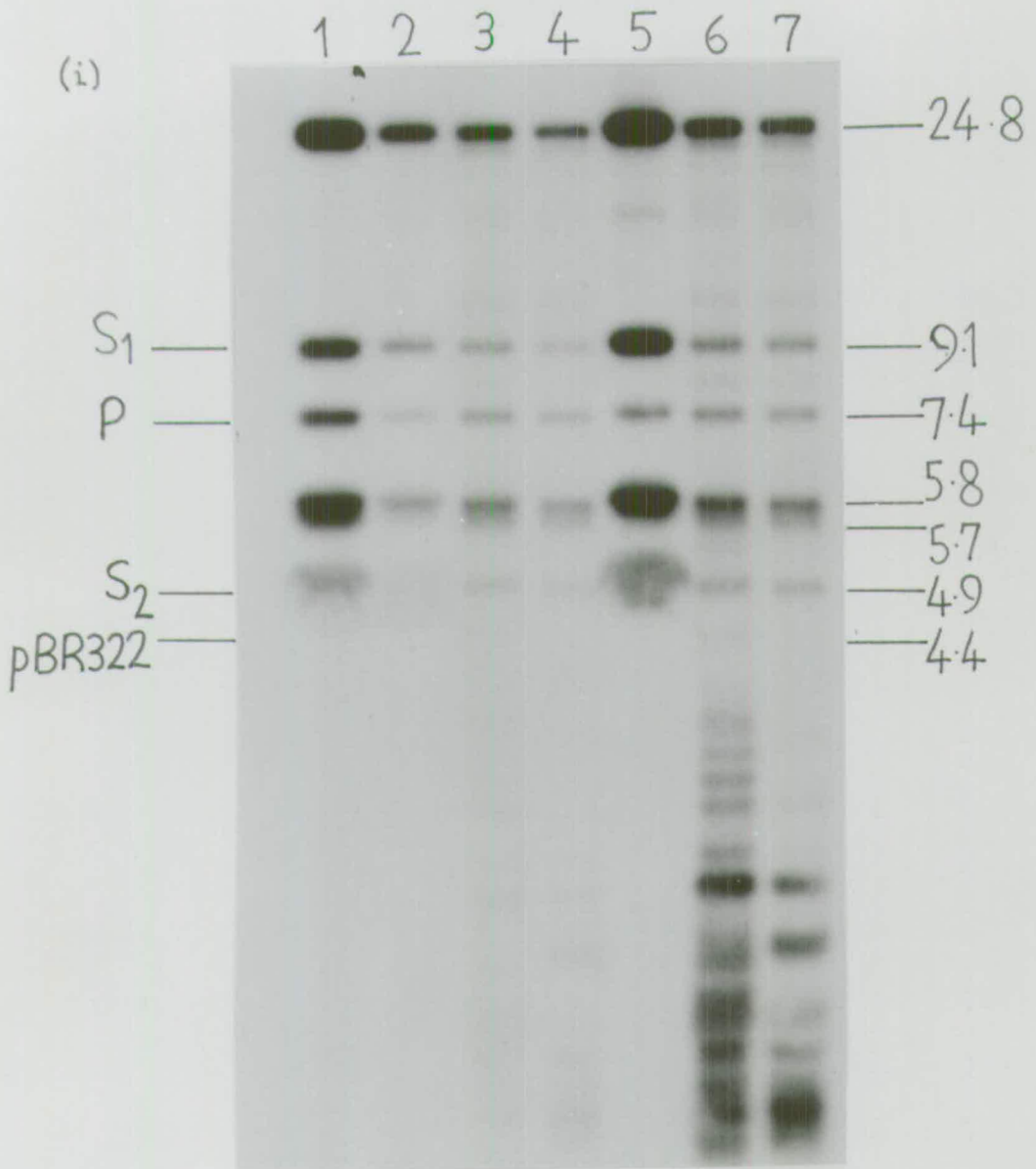


figure 5.2 and figure legend)

In contrast to this result, the levels of hemimethylated palindrome containing DNA had not decreased with time. The ratio of P to  $S_1$  for the hemimethylated DNA is 1.3 for the 7 minute infection and 0.83 for the 15 minute infection. The relative recovery ( $R_H$ ) of the hemimethylated palindrome containing supercoils (compared to the  $P/S_1$  ratio of 0.67 for the supercoils extracted from the lysogen), was therefore 2.0 at 7 minutes and 1.3 at 15 minutes. At first the value of 2.0 for the 7 minute infection appears anomalously high, however a comparison of the DNA before and after DpnI digestion provides an explanation. The band P is approximately the same intensity before and after DpnI digestion (lanes 3 and 4 in figure 5.2), which indicates that almost all of the palindrome containing molecules have undergone at least one round of replication by 7 minutes and there are virtually no unreplicated (fully methylated) molecules remaining. However  $S_1$  is less intense after DpnI digestion suggesting that some unreplicated control molecules remain at 7 minutes. By 15 minutes they have replicated as the intensity of  $S_1$  before and after DpnI digestion is approximately the same. The ratio of P to  $S_1$ , for the 7 minute infection time, before DpnI digestion is 1.0 after digestion it is 1.3. This can be expressed in the following manner:

$$(P_M + P_{HM}) / (S_M + S_{HM}) = 1.0 \dots\dots\dots(1)$$

$$P_{HM} / S_{HM} = 1.3 \dots\dots\dots(2)$$

where  $P_M$  and  $S_M$  are the amounts of fully methylated



DNA and  $P_{HM}$  and  $S_{HM}$  are the amounts of hemimethylated DNA of DRL110 and JMC249 respectively.

Rearranging equation (2) gives:

$$S_{HM} = P_{HM}/1.3$$

$$S_{HM} = 0.77 P_{HM} \dots\dots\dots(3)$$

Assuming  $P_M$  to be zero and rearranging equation (1)

$$S_M + S_{HM} = P_{HM} \dots\dots\dots(4)$$

Substituting the value of  $S_{HM}$  from equation (3)

into equation (4) gives:

$$S_M + 0.77 P_{HM} = P_{HM}$$

$$S_M = 0.23 P_{HM}$$

This is approximately the amount of fully methylated JMC249 DNA remaining after 7 minutes of growth, expressed in terms of  $P_{HM}$ . Had this DNA replicated it would have contributed twice this amount of hemimethylated DNA to band  $S_{HM}$  i.e.  $0.46 P_{HM}$ . The ratio of  $P_{HM}/S_{HM}$  of 1.3 previously calculated for 7 minutes can now be corrected to allow for the unreplicated control DNA as follows:

$$P_{HM}/S_{HM} \text{ (new)} = P_{HM}/(S_{HM} \text{ (old)} + 0.46P_{HM})\dots\dots(5)$$

Substituting the values from (3)

$$P_{HM}/S_{HM} \text{ (new)} = P_{HM}/(0.77P_{HM} + 0.46P_{HM})\dots\dots(6)$$

This gives a corrected  $P_{HM}/S_{HM}$  ratio of 0.81. The corrected relative recovery of hemimethylated palindrome DNA ( $R_H$ ) at 7 minutes (relative to the lysogen  $P/S_1$  ratio of 0.67) was therefore 1.2 in close agreement with the  $R_H$  of 1.3 at 15 minutes and the theoretical  $R_H$  of 1.

The ratios of  $S_1$  to  $S_2$  (both bands unique to JMC249, whose ratio should therefore be the same in all tracks) could be calculated for tracks 3,4,6 and 7 and

were found to be 2.1, 1.6, 1.8 and 1.8 respectively. These ratios differ by a maximum factor of 1.3 (2.1/1.6) showing that the interlane variance is low but sufficient to account for the deviation of the  $R_H$  from the theoretical value of 1.

These results indicate that destruction of the palindrome containing DNA does not occur, but rather that the palindrome containing DNA is replicated at a slower rate than the palindrome free DNA. The number of rounds of replication that each phage had undergone by fifteen minutes could be estimated by comparing the amounts of total and hemimethylated DNA for each phage. The ratio of  $S_1$  from the total DNA to  $S_1$  for the hemimethylated DNA was approximately 10 at fifteen minutes, this was multiplied by 2 to compensate for the difference in loading volumes (see figure 5.2 legend) giving a ratio of total JMC249 to hemimethylated JMC249 DNA of 20. From table 5.1 which shows the theoretical ratio of total to hemimethylated DNA following each round of replication, it could be estimated that the control phage had undergone slightly over 5 rounds of replication. The corresponding ratio for the DRL110 DNA was approximately 4, indicating that the palindrome had undergone only 3 rounds of replication in this time.

#### 5.4 Infection of a *recBC sbcB sbcC dam*<sup>-</sup> strain with *dam* modified DRL110 and JMC249

There is no, or very little, reduction of palindrome containing supercoils following the infection

**Table 5.1**

The number of molecules of each type (fully methylated, hemimethylated and unmethylated) formed from a single fully dam methylated molecule following each round of replication in a dam<sup>-</sup> strain.

No. of rounds of replication	0	1	2	3	4	5	6
Total no. of molecules	1	2	4	8	16	32	64
No. of fully methylated	1	0	0	0	0	0	0
No. of hemimethylated	0	2	2	2	2	2	2
No. of unmethylated	0	0	2	6	14	30	62
Ratio of total to hemimethylated molecules	-	1	2	4	8	16	32
Ratio of unmethylated to hemimethylated molecules	-	-	1	3	7	15	31

of a recBC sbcB sbcC strain (see chapter 4). This was assumed to represent a genuine alleviation of the problem causing inviability, however an alternative explanation is that the supercoils extracted from recBC sbcB sbcC strains consist of unreplicated or poorly replicated DNA, with most actively replicating supercoils having switched to rolling circle replication in the 10 minute infection time. If this were the case, a reduction of actively replicating palindrome molecules could pass unnoticed.

In order to dispel this hypothesis and to confirm that the palindrome containing phage does not replicate slowly in recBC sbcB sbcC strains, an infection of a recBC sbcB sbcC dam<sup>-</sup> strain (JL32) was performed, with equal numbers of dam modified DRL110 and JMC249 phage (m.o.i. of 2 for each). The DNA was extracted and the phage supercoils prepared on a CsCl/EtBr gradient following 7 and 15 minutes of exponential growth. A simultaneous 7 minute infection of a rec<sup>+</sup> dam<sup>-</sup> lambda lysogen (JL33) was also performed. Phage supercoils were digested with EcoRI to generate fragments specific to each phage and portions of phage supercoils from the non lysogen infection were further digested with MboI or MboI + DpnI in the manner described in the previous two sections. As before digested samples were electrophoresed on an agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled lambda DNA.

As can be seen from figure 5.3, both the total and the hemimethylated palindrome containing phage DNA was recovered well. The ratio of P (unique to DRL110) to

**Figure 5.3**

JMC249 + DRL110 supercoiled DNA recovered following infection of a rec<sup>+</sup> dam<sup>-</sup> lysogen and a recBC sbcB sbcC dam<sup>-</sup> non lysogen. DNA recovered from the lysogen digested with EcoRI (lane 1); the non lysogen after 7 minutes digested sequentially with EcoRI (lane 2) + MboI (lane 3) + DpnI (lane 4) and the non lysogen after 15 minutes digested sequentially with EcoRI (lane 5) + MboI (lane 6) + DpnI (lane 7). EcoRI restriction fragments from lanes 1, 2 and 5 therefore consist of total DNA (fully methylated + hemimethylated + unmethylated), from lanes 3 and 6 of fully and hemimethylated DNA and from lanes 5 and 7 of hemimethylated DNA only. Twice as much DNA was loaded in lanes 3,4 and 6,7 compared to lanes 2 and 5 respectively. Bands P and S<sub>1</sub> were used to quantify the yields of the two phage DNAs. Fragment sizes are given in kilobase pairs. The pBR322 band was used to confirm digestion in the manner previously described for the rec<sup>+</sup> dam<sup>-</sup> infection (figure 5.2 (ii)).



$S_1$  (unique to JMC249) in the total DNA was found to be 1.2 for the DNA extracted from the lysogen, 0.82 for the DNA extracted after 7 minutes of growth in the recBC sbcB sbcC non lysogen and 0.65 for the DNA extracted after 15 minutes of growth in this strain. This gives a relative recovery ( $R_T$ ) of total palindrome DNA from the non lysogen infection compared to the lysogen infection of 0.7 at 7 minutes and 0.5 at 15 minutes, again suggesting that the palindrome has a slightly deleterious effect on the supercoil level even in recBC sbcB sbcC strains. As for the rec<sup>+</sup> dam<sup>-</sup> experiment the ratios of  $S_1$  to  $S_2$  could not be calculated in these tracks due to the anomalous migration of the unmodified plasmid DNA.

The recovery of hemimethylated palindrome DNA,  $R_H$ , compared to the recovery of palindrome DNA from the lysogen was calculated to be 1.4 at 7 minutes and 1.4 at 15 minutes, showing that there is no loss of hemimethylated palindrome containing DNA. The ratios of  $S_1$  to  $S_2$  were calculated for lanes 3,4,6 and 7 and found to differ by a maximum factor of 1.2, showing that the interlane variance is low.

The ratio of total control phage DNA to hemimethylated control phage DNA was calculated to be approximately 26 after 15 minutes of growth, indicating that this phage had undergone between 5 and 6 rounds of replication in this time (see table 5.1). This is about the same number of rounds of replication that JMC249 had undergone in the rec<sup>+</sup> dam<sup>-</sup> strain, indicating that the supercoils extracted from a recBC sbcB sbcC are not under



replicated. The ratio of total palindrome containing phage DNA to hemimethylated palindrome containing DNA was calculated to be approximately 11, indicating that DRL110 has undergone between 4 and 5 rounds of replication in the 15 minute infection time.

### 5.5 Infection of a *recA* strain with *EcoRI* modified DRL110 and JMC249

This experiment was carried out to confirm the results of the *rec*<sup>+</sup> *dam*<sup>-</sup> infection and to extend the observations made in this experiment to a strain which was not *dam*<sup>-</sup> and possessed a different genetic background. A *recA* strain was used to allow longer infection times.

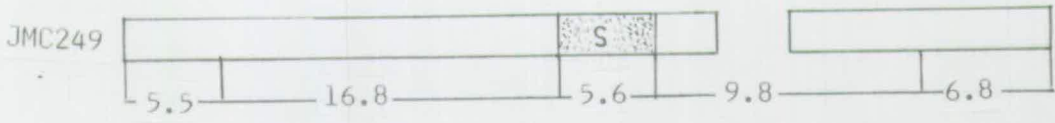
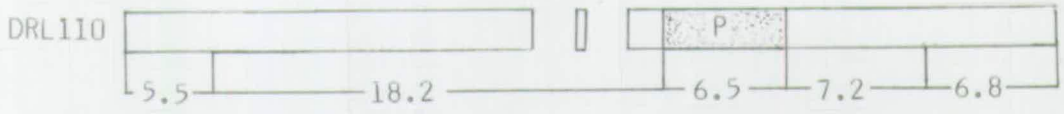
Infection of the *recA* strain (JL4) was carried out using equal numbers of *EcoRI* modified DRL110 and JMC249 phage (m.o.i of 2 for each phage). The DNA was extracted and the phage supercoils prepared on a CsCl/EtBr gradient following 10 and 60 minutes of exponential growth. A simultaneous 10 minute infection of a *recA* lysogen (JL9) was also performed. A proportion of the isolated phage supercoils were digested with *Bam*HI, which generates fragments specific to each phage (figure 5.4), electrophoresed on an agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled lambda DNA. As can be seen from figure 5.4, the amount of total palindrome containing DNA has decreased with time, giving a relative recovery of palindrome containing phage DNA ( $R_T$ ), compared to the lysogen infection, of 0.26 at 10 minutes and 0.11 at 60 minutes.

**Figure 5.4**

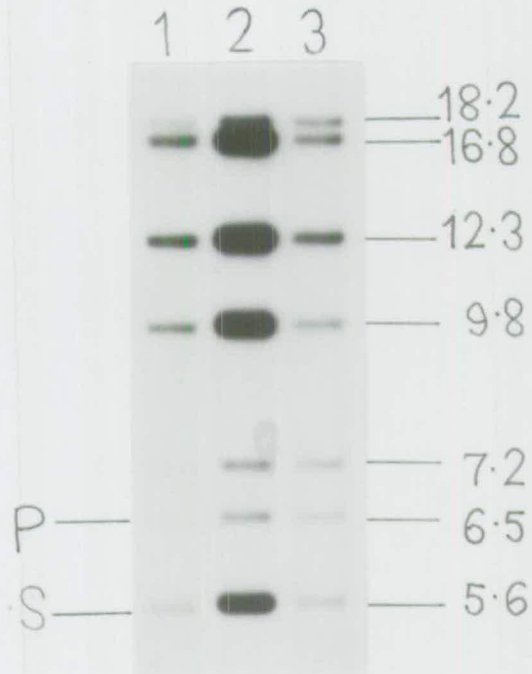
(i) BamHI restriction maps of DRL110 and JMC249. Fragment sizes are given in kilobase pairs. Deletions are shown as interruptions in the genome.

(ii) DNA recovered following infection of a recA lysogen and non lysogen with EcoRI modified phage. BamHI digests of supercoiled DNA of DRL110 + JMC249 recovered from a recA lysogen (lane 3) and non lysogen after 10 (lane 1) and 60 (lane 2) minutes of growth.

(i)



(ii)



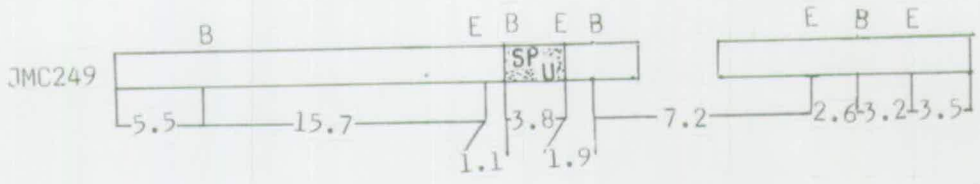
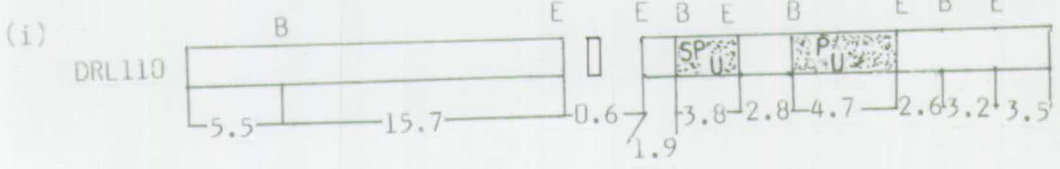
To distinguish methylated DNA from unmethylated DNA a double digest of the phage supercoils with BamHI and EcoRI was performed. This generates fragments specific to methylated and unmethylated DNA, as the former are resistant to EcoRI cleavage. Fully methylated DNA can not be distinguished from hemimethylated DNA, as both are resistant to cleavage. However the dam<sup>-</sup> experiments show that there are few fully methylated molecules in the DNA extracted from cells where it is replicating, particularly at the later infection times. Following digestion, the fragments were run on an agarose gel, transferred to nitrocellulose and hybridised to radioactively labelled lambda DNA. A unique unmethylated control band could not be distinguished on the autoradiograph, however a comparison of the intensity of the band SP<sub>U</sub> (a doublet of bands from both phage), with the band P<sub>U</sub>, provided an estimation of the amount of unmethylated control DNA.

It can be seen from figure 5.5, that the DNA extracted from the lysogen, showed no digestion with EcoRI, this shows that both infecting phage stocks were totally methylated. While there is a large reduction in the level of DRL110 supercoils in the unmethylated DNA extracted from the non lysogen (c.f. SP<sub>U</sub> and P<sub>U</sub>), methylated DRL110 supercoils are recovered efficiently. Compared to the lysogen, the relative recovery of methylated palindrome containing DNA ( $R_M$ ) was 0.48 at 10 minutes and 0.56 at 60 minutes. These results show that the amount of methylated DNA has not decreased between 10 and 60 minutes of growth, although the phage have clearly

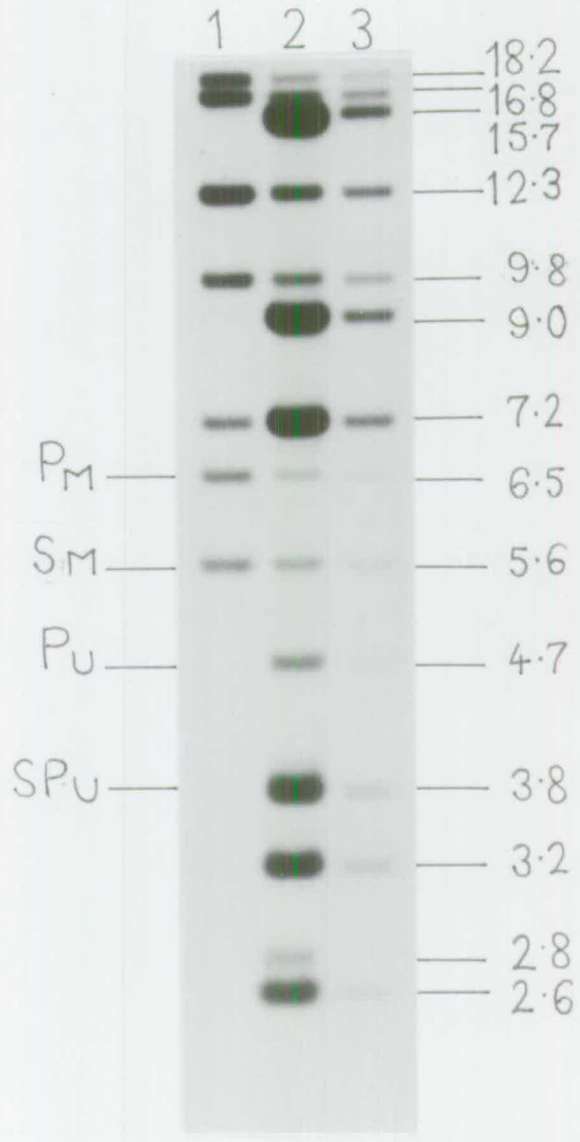
**Figure 5.5**

(i) Restriction maps of DRL110 and JMC249 showing BamHI and EcoRI sites, indicated by the letters B and E respectively. Fragment sizes are given in kilobase pairs. Deletions are shown as interruptions in the genome.

(ii) DNA recovered following infection of a recA lysogen and non lysogen with EcoRI modified phage. BamHI and EcoRI double digests of supercoiled DNA of DRL110 + JMC249 recovered from a recA lysogen (lane 1) and non lysogen, after 10 (lane 3) and 60 (lane 2) minutes of growth.



(ii)



been actively replicating in this time as indicated by the increase in the amount of unmethylated DNA.

These results confirm the results of the rec<sup>+</sup> dam<sup>-</sup> experiment and indicate that destruction is not occurring. The relative recovery of methylated DRL110 from the non lysogen compared to the lysogen is slightly low compared to the expected ratio of 1. This may be an anomalous result, or it may be because some unreplicated DRL110 molecules persist. Alternatively it may be a genuine decrease and this will be considered further in section 5.6.

From the ratio of unmethylated control DNA to methylated DNA of approximately 15 after 60 minutes of replication it was calculated that the control phage had undergone about 5 rounds of replication in this time. This is less replication than would perhaps be expected, as the control phage had undergone as many rounds in the 15 minute infections of the dam<sup>-</sup> rec<sup>+</sup> and recBC sbcB sbcC strains. The ratio of unmethylated palindrome DNA to methylated DNA was approximately 3 indicating that the palindrome phage had undergone only 3 rounds of replication in the 60 minute infection time.

## 5.6 Discussion

The results show that there is no decrease in the levels of palindrome containing molecules with 'input' (methylated) strands with time. This makes it unlikely that the apparent 'loss' of actively replicating molecules conferred by the presence of the palindrome is due to

destruction of the DNA and indicates that it is due instead to the palindrome causing a decrease in the rate of formation of newly synthesized DNA strands. These results argue against double strand cleavage hypotheses, such as those which postulate that the palindrome forms a cruciform which is cleaved by nucleases, as if it were a Holliday junction, because these hypotheses predict destruction of the phage DNA.

The results are perhaps not sensitive enough to rule out a 'single-strand' destruction hypothesis, postulating that one strand of the phage DNA is destroyed during replication. For example, a hairpin could form occasionally on the lagging strand, but not on the leading strand, during replication, followed by cleavage and degradation of this strand. This mechanism would mimic slow replication in terms of supercoil loss, except that it would result in a 50% decrease of hemimethylated molecules, due to destruction of all the input methylated lagging strands. Such a decrease was seen for the recA infection, but not for the rec<sup>+</sup> dam<sup>-</sup> infection. It is possible, but unlikely, that a 50% decrease of hemimethylated palindrome containing molecules did occur in the rec<sup>+</sup> dam<sup>-</sup> strain, but the method used to monitor supercoil loss was not sufficiently sensitive to detect it. It is more plausible however that the rec<sup>+</sup> dam<sup>-</sup> infection result which showed no decrease in the hemimethylated palindrome containing molecules is the correct one, as in the recA infection fully and hemimethylated DNA could not be distinguished and the 50%



decrease could be explained in terms of unreplicated palindrome carrying molecules persisting (c.f. the apparent 33% decrease in control molecules after 7 minutes growth in the rec<sup>+</sup> dam<sup>-</sup> strain, due to this effect). Therefore it is unlikely that any destruction of palindrome containing DNA occurs.

The palindrome seems instead to cause slow replication of its carrier molecule. From the results it appears that the palindrome containing DNA is replicated at about 60% of the rate of the control DNA in rec<sup>+</sup> and recA cells and at about 80% of the rate of the control DNA in recBC sbcB sbcC cells. This may be due to a direct effect of the palindrome on replication, such as hairpin formation impeding the passage of DNA polymerase III, or due to an indirect effect, such as extrusion of the palindrome into a cruciform delaying supercoiling of the daughter molecules, so that replication is slow to reinitiate. If the hairpin impediment hypothesis is correct, then the hairpin must be capable of delaying DNA synthesis without preventing it completely; that is DNA polymerase III must be able to replicate through the palindromic region eventually. Pausing of DNA polymerase III core and holoenzyme at hairpins has been demonstrated in vitro and it has been shown that these enzymes were able to progress through these regions if additional enzyme and longer incubation times were used (LaDuca et al., 1983). This hypothesis is attractive because it means that a common cause of both palindrome instability and palindrome mediated inviability can be postulated. Pausing

by DNA polymerase III at hairpins in the replication fork could lead to both instability, by promoting separation and reassociation elsewhere of the nascent DNA strand end (see chapter 3 for a full account of the mechanism) and inviability, by significantly slowing down DNA replication.

In recBC sbcB sbcC cells, the slow replication seems to be largely overcome. As stated previously it is the absence of the sbcC gene product which is important in determining viability of palindrome carrying replicons. As nothing else is known about the sbcC gene product, apart from the fact that its absence is needed to fully suppress the mitomycin C sensitivity, lethal sectoring and recombination deficiency associated with the recBC genotype, it is difficult to make any concrete speculations about its role in palindrome mediated inviability. It is possible that it promotes the formation of hairpins in the replication fork, possibly by having some sort of unwinding activity which extrudes palindromes into cruciforms preceding replication. Hairpins may form only rarely in the replication fork in the absence of the sbcC gene product, but if the palindrome is pre-extruded into a cruciform ahead of the replication fork, by this product, this structure may take time to be destabilized and so delay replication. Alternatively, the sbcC gene product may increase the sensitivity of DNA polymerase to hairpin impediments. The four catalytically active forms of E.coli DNA polymerase (core enzyme, DNA polymerase III', DNA polymerase III\* and the holoenzyme) which

possess different combinations and amounts of subunits, have been observed to show markedly different abilities to progress through template specific barriers in vitro (LaDuca et al.,1983). It is possible that the sbcC gene products somehow affects the synthesis of, or interacts with, a DNA polymerase III subunit in vivo, altering the capacity of the enzyme to overcome hairpin impediments. There are many other ways in which the sbcC gene product could affect palindrome mediated inviability and a greater knowledge of the nature of this product, should provide a deeper insight into the mechanism of inviability.

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## Appendix I



# In vivo loss of supercoiled DNA carrying a palindromic sequence

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**Summary.** Interest in the fate of long palindromic DNA sequences in *E. coli* has been kindled by the observation that their inviability is overcome in *recBC sbcB* strains and that these hosts permit the construction of DNA libraries containing long palindromic sequences present in the human genome. In this paper we show that a reduction in the level of intracellular supercoiled DNA occurs as the result of the presence of a 530 bp palindrome in bacteriophage lambda. This reduction occurs in *Rec<sup>+</sup>* and *recA* strains but not in strains lacking exonucleases V and I (*recBC sbcB*). However, the DNA must be active (not repressed) for this reduction to be observed, since it is not seen in a *Rec<sup>+</sup>* host lysogenic for phage lambda. These results argue against two hypotheses: firstly, that the palindrome causes inviability solely by interfering with packaging, and secondly, that it does so solely by interfering with recombination. Conversely, these results suggest that a feature of active monomeric DNA (probably its replication) is involved in inviability.

**Key words:** Palindrome – Lambda – *recBC sbcB* – *E. coli*

## Introduction

In *Escherichia coli* palindromes of greater than approximately 500 base pairs total length show two features. Firstly, they are lethal to the carrier replicon (inviability) and secondly, they undergo *RecA*-independent deletion at varying frequencies (instability) leaving the carrier replicon viable (Collins 1981; Collins et al. 1982; Hagan and Warren 1982; Mizuuchi et al. 1982; Hagan and Warren 1983; Leach and Stahl 1983). The properties of only a few palindromes smaller than 500 base pairs in length have been studied. Lilley (1981) reported the failure to clone a palindrome of 260 bp. This appears to be due to the high instability of this replicon rather than to inviability. A palindrome of 147 bp derived from the origin of replication of SV40 is stable in *E. coli* (Bergsma et al. 1982) but may confer a small degree of inviability since when re-cloned into pBR322 it reduces the copy number of the plasmid (Warren and Green 1985). Furthermore, it increases the ratio of monomers to multimers of the plasmid. These properties have also been shown for an unrelated 146 bp palindrome but not for its 114 bp derivative. Instability has, however, been shown for a shorter palindrome of 68 bp (Courey and Wang 1983). In addition to these effects, in

the presence of chloramphenicol, a plasmid carrying a 58 bp palindrome accumulates eye structures presumed to be caused by strand switching of DNA polymerase I (Bolivar et al. 1977).

Leach and Stahl (1983) have shown that a lambda phage carrying a 3,200 bp palindrome is viable in *recBC sbcB* strains but inviable in *Rec<sup>+</sup>* strains. This palindrome is, however, unstable in the *recBC sbcB* cells where it gives rise to shorter palindromes of approximately 500–600 bp. The latter phage are both viable and stable in *recBC sbcB* cells but inviable in *Rec<sup>+</sup>*. These results imply that exonucleases V and I (the products of the *recBC* and *sbcB* genes) are either directly or indirectly responsible for the loss of palindromic DNA. Recently it has been shown that when *recBC sbcB* hosts are used for the primary screening of human DNA libraries, 9% of the clones obtained do not plate on *Rec<sup>+</sup>* hosts and many of these carry palindromic DNA sequences (Wyman et al. 1985).

Two observations have been made relating to the effect of *recBC sbcB* mutations on plasmid-borne palindromes. Firstly, Collins et al. (1982) have shown that *recBC sbcB* hosts reduce the frequency of precise excision of palindromes bounded by short direct repeats. Secondly, a plasmid carrying a 206 bp imperfect palindrome which is unstable in wild type hosts can be stably propagated in a *recBC sbcB recF* host (Boissy and Astell 1985).

Two hypotheses have been advanced to explain instability. The first is that during replication, hairpin loops are formed on the lagging strand and that the replication machinery occasionally jumps across them, creating deletions (Collins 1981; Courey and Wang 1983). The second is that recombination occurs between short direct repeats within or bounding the palindrome, leading to deletion of the intervening DNA (Collins et al. 1982). Explanations for inviability have mainly been based on the effects or possible consequences of cruciform extrusion. These include loss of supercoiling (Lilly 1981), a block to replication (this paper), and cleavage of the base of the cruciform as though it were a Holliday junction (Mizuuchi et al. 1982; Leach and Stahl 1983). Other explanations such as the formation of double-stranded mRNA species and of unresolved recombination intermediates have also been suggested (Warren and Green 1985). In addition, it is possible that the formation of hairpin loops in the lagging strand following DNA replication, also suggested to cause instability, can block replication (Bolivar et al. 1977).

It has been shown in vitro that palindromic DNA can adopt a cruciform conformation (Gellert et al. 1979; Lilley 1981; Panayotatos and Wells 1981; Mizuuchi et al. 1982).

Table 1. Bacterial strains

Number	Relevant genotype	Lambda lysogen
JC9937	<i>rec</i> <sup>+</sup>	—
JC9387	<i>recB21 recC22 shcB15</i>	—
DL187	<i>rec</i> <sup>+</sup>	+
DL188	<i>recB21 recC22 shcB15</i>	+
W3101 RecA <sup>-</sup>	<i>recA13</i>	—

Strains JC9937, JC9387, DL187 and DL188 are derivatives of AB1157 Su<sup>-</sup>, JC9387 and JC9937 were isolated in the laboratory of A.J. Clark and obtained from F. Stahl. DL187 and DL188 were isolated for this work by lysogenisation with JMCO-82 (*λred3 gam210 chiA131*). W3101 RecA<sup>-</sup> was isolated in the laboratory of N. Franklin and obtained from N. Murray. The genotypes of AB1157 and W3101 are F<sup>-</sup> DEL (*gpt-proA*) 62 *argE3 his-4 leu-6 thr-1 ara-14 galK2 lacY1 xyl-5 mtl-1 thi-1 supE44 rpsL31 tsx33* and F<sup>-</sup> *galT1* respectively

These studies have, however, not clarified whether this could be the cause of inviability in vivo. The kinetics of cruciform extrusion under physiological conditions can be so slow that the transition is unlikely to occur unless catalysed in vivo (Courey and Wang 1983; Gellert et al. 1983). However, this is not always the case, since a poly-AT palindrome forms a cruciform rapidly (Greaves et al. 1985). Also small palindromes (68 and 66 base pairs) which can adopt a cruciform structure in vitro do not do so in vivo (Courey and Wang 1983; Sinden et al. 1983).

We have used a *recBC sbcB* strain to prepare bacteriophage lambda particles carrying a 530 base pair palindrome and used them to study the fate of this DNA in hosts where the phage is not viable.

## Materials and methods

**Bacterial strains.** The bacterial strains used are described in Table 1.

**Lambda phage strains.** Lambda phages carrying a palindrome of approximately 530 bp were constructed by in vivo deletion from a 3.2 kb palindrome. The 3.2 kb palindrome is the same as that previously described as a 1.6 kb palindrome by Leach and Stahl (1983). Throughout this paper the total lengths, instead of repeat lengths, have been used in contrast to the earlier work. DNA from these particles was extracted and snap-backs of single strands were viewed under the electron microscope to confirm the presence of the palindrome and to measure its approximate length. These studies showed that no loop could be detected between the two inverted repeats of the palindrome, suggesting that any asymmetry at its centre was small (0–50 bp approximately) (see Fig. 1). This phage (DRL107) plates at a frequency of 10<sup>-4</sup> on Rec<sup>+</sup> hosts when compared to its plating on *recBC sbcB* strains. A derivative of this phage (DRL110) carrying an amber mutation in the *S* gene was also constructed so that experiments could be conducted without lysis of the host cell. These phage strains and others used in this work are shown in Fig. 2.

**Electron microscopy.** Single strand snapbacks were prepared with DNA extracted from phage particles by denaturation

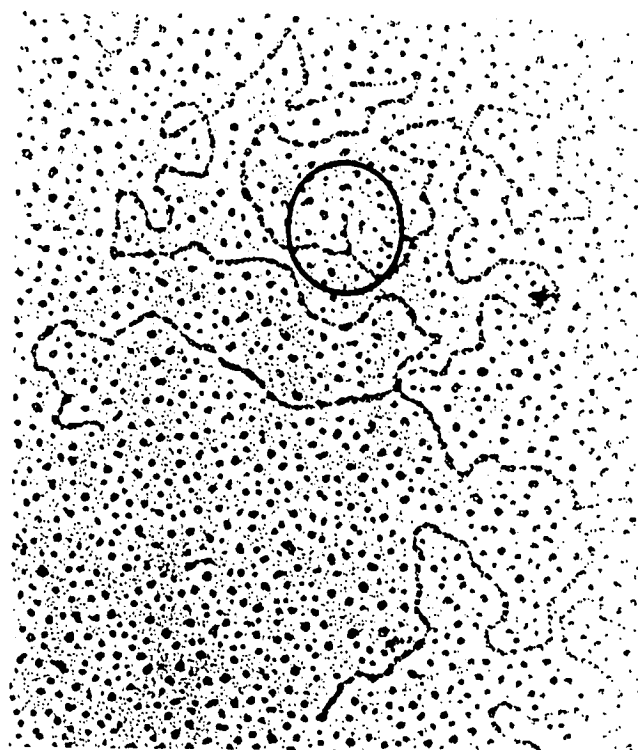


Fig. 1. Electron micrograph of a single strand of DRL107 showing (within the circle) the palindrome as a double strand snap-back

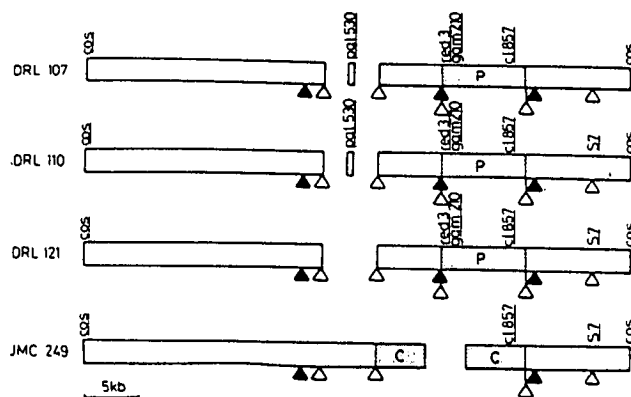


Fig. 2. DNA structure and genotype of the lambda phages used. *EcoRI* sites are represented as open triangles while *SmaI* sites are represented as closed triangles. Deletions ( $\Delta B$  in DRL107, DRL110 and DRL121,  $\Delta spi-6$  in JMC249) are shown as interruptions in the Figure. *red3* is a missense mutation, *gam210* and *S7* are amber mutations and *c1857* is a temperature-sensitive mutation. *EcoRI* restriction fragments P and C are shaded to indicate they were used to quantitate the recovery of supercoiled DNA molecules

in 0.1 M NaOH followed by neutralisation for 15 min before spreading by the method of Davis et al. (1971). Lengths were measured using a Ferranti Cetec digitiser.

**Extraction of supercoiled circles of lambda DNA.** Cells to be infected were grown at 37°C to  $2 \times 10^8$  in L broth supplemented with 0.2% maltose and 5 mM MgSO<sub>4</sub>. They were then starved by shaking for 30 min at 37°C in 10 mM Tris 10 mM MgSO<sub>4</sub>. Infection was allowed to occur at a multiplicity of infection of approximately 2 for each phage for 30 min at room temperature. The culture was then di-

luted 5-fold into prewarmed L broth at 37° C and shaken vigorously for 10, 60 or 90 min (see Results). To stop further growth of the cells they were placed at 0° C in an ice water bath. The cells were then washed twice with 0° C L broth to remove unadsorbed phage particles and resuspended in 50 mM Tris 25% sucrose pH8. Supercoiled DNA was extracted using the cleared lysate method (Reuben et al. 1974) followed by phenol extraction and centrifugation in a CsCl-ethidium bromide gradient for 16 h at 200,000 g in a vertical rotor. The amount of supercoiled DNA prepared was not sufficient for a band to be visible when illuminated with long wave UV light. However, the material of higher density than the visible band of relaxed DNA (i.e. from the part of the gradient where supercoils were expected to run) was removed, the ethidium bromide extracted with n-butanol saturated with 5 M NaCl, precipitated two times with ethanol in the presence of 5 µg/ml tRNA (from Boehringer, Mannheim, FRG) and run on a 0.7% agarose gel. At this stage the band of supercoiled lambda circles could be seen.

**Restriction analysis and southern hybridisation.** Supercoiled lambda DNA was digested to completion with *Eco*RI (from Amersham, U.K. or Boehringer, Mannheim, FRG) and the fragments were heated to 70° C for 10 min to denature the cohesive ends of any contaminating linear DNA and run on a 0.7% agarose gel beside control tracks with *Eco*RI digested linear, phage particle DNA, treated in parallel. The presence of bands generated by denaturation of the cohesive ends in the tracks with linear DNA and the absence of these bands from the tracks with circular DNA was taken as evidence that contamination of the supercoils with linear DNA was undetectable.

The fragments were then transferred to nitrocellulose and probed with <sup>32</sup>P labelled lambda DNA according to the method of Smith and Summers (1980).

**Densitometry.** The amounts of material present in bands was estimated using a Vitatron densitometer followed by tracing of the peaks with a Ferranti Cetec digitiser. Several exposures of the film were taken to ensure that the response was approximately linear.

## Results

### Infection of lysogens

In order to test whether DNA carrying a palindrome was destroyed following intracellular supercoiling but in the absence of replication or extensive transcription, cells lysogenic for phage lambda were infected with the palindrome-containing phage. Following injection of the lambda genome, circularisation and supercoiling occur but the DNA remains repressed by the *cI* repressor.

*Rec*<sup>+</sup> and *recBC sbcB* strains lysogenic for bacteriophage lambda (DL187 and DL188 respectively) were infected with DRL107, which carries the palindrome, and JMC249, a palindrome-free control. After 90 minutes of exponential growth the cells were lysed and supercoiled DNA prepared on a CsCl-ethidium bromide gradient. Since the two DNA molecules have indistinguishable mobilities on agarose gels, the supercoiled fraction was subjected to digestion with the restriction enzyme *Eco*RI. As DRL107 and JMC249 carry deletions in different parts of their ge-

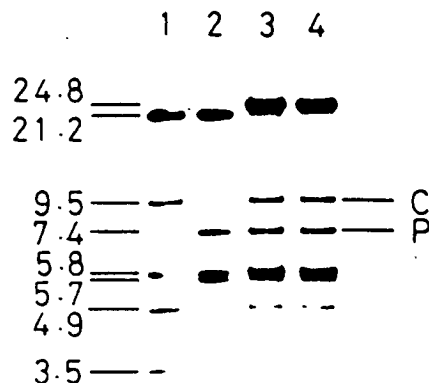


Fig. 3. DNA recovered from *Rec*<sup>+</sup> and *recBC sbcB* lysogens. *Eco*RI digests of: Linear DNA from JMC249 (lane 1) and DRL107 (lane 2) extracted from phage particles; and supercoiled DNA of JMC249 + DRL107 recovered from *recBC sbcB* lysogen DL188 (lane 3) and *Rec*<sup>+</sup> lysogen DL187 (lane 4). Bands are labelled according to their size (in kilobase pairs) and bands P and C were used to compare the yields of the two supercoiled DNAs (see Fig. 2)

nomes, fragments specific to each molecule are generated by cleavage. The DNA was electrophoresed on an agarose gel and then in order to increase the sensitivity of the experiment the restriction fragments were transferred to a nitrocellulose filter and hybridised to <sup>32</sup>P labelled λ DNA.

As can be seen in Fig. 3, both DNA molecules are recovered approximately equally well in *recBC sbcB* and *Rec*<sup>+</sup> lysogenic cells (lanes 3 and 4) indicating that the palindrome does not cause the molecule in which it is located to be lost from the supercoiled fraction. The yields of the two DNAs were quantified by densitometry and the relative recovery of palindrome containing DNA was calculated as:

$$R = (P/C \text{ Rec}^+) / (P/C \text{ recBC sbcB})$$

where P and C represent the intensities of the bands labelled P and C in Fig. 3. This gives a value of  $R = 1.1$  which is not significantly different from a relative recovery of  $R = 1.0$  indicating equal yields of the two DNA types.

### Infection of non-lysogens

To test whether the presence of a palindrome caused loss of supercoiled DNA under conditions where that DNA was active, the above experiment was repeated using the non-lysogenic strains JC9937 (*Rec*<sup>+</sup>) and JC9387 (*recBC sbcB*). In addition, two modifications to the experiment were made. Firstly, following injection, the cells were allowed to grow exponentially for only 10 minutes to minimise the amount of rolling-circle replication in the *recBC sbcB* strain and to prevent DNA packaging into phage heads. Secondly the lambda phages used (DRL110 and JMC249) both carried the *Sam7* mutation to ensure that no phage-induced lysis of the cells occurred.

As can be seen in Fig. 4 the ratio of supercoiled DNAs of DRL110 to JMC249 extracted from *Rec*<sup>+</sup> infected cells is lower than that of the same two DNAs extracted from *recBC sbcB* infected cells ( $R = 0.33$ ). No loss of palindrome containing DNA was detected in the *recBC sbcB* cells when the recovery of bands P and C was compared following infection of non lysogenic and lysogenic bacteria JC9387 and DL188 (data not shown).



Fig. 4. DNA recovered from  $Rec^+$  and *recBC sbcB* non-lysogens. *EcoRI* digests of: supercoiled DNA of DRL110+JMC249 recovered from  $Rec^+$  JC9937 (lane 1) and *recBC sbcB* strain JC9387 (lane 2); and linear DNA from DRL110 (lane 3) and JMC249 (lane 4) extracted from phage particles. Bands are labelled according to their size (in kilobase pairs) and bands P and C were used to compare the yields of the two supercoiled DNAs (see Fig. 2). The poor recovery of band P in lane 2 was due to an inequality of the multiplicities used in this experiment

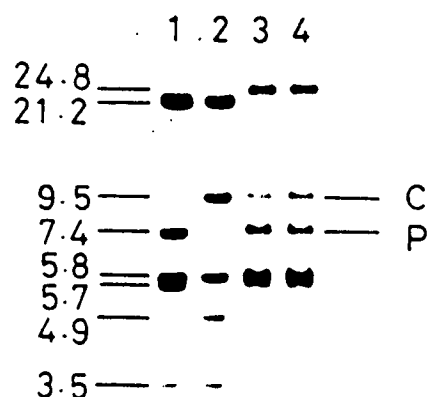


Fig. 5. Control experiment using phage without a palindrome. *EcoRI* digest of: linear DNA from DRL110 (lane 1) and JMC249 (lane 2) extracted from phage particles; and supercoiled DNA of DRL121+JMC249 recovered from  $Rec^+$  strain JC9937 (lane 3) and from *recBC sbcB* strain JC9387 (lane 4). Bands are labelled according to their size (in kilobase pairs) and bands P and C were used to compare the yields of the two supercoiled DNAs (see Fig. 2)

To confirm that this loss of supercoiled DNA was due to the presence of the palindrome in DRL110 and not to some other difference between the two DNAs a control experiment was carried out using DRL121, a phage which lacks the palindrome but is otherwise identical to DRL110. The results of this experiment are shown in Fig. 5 where it can be seen that supercoiled DRL121 DNA is efficiently recovered from both  $Rec^+$  and *recBC sbcB* hosts. In fact the DNA of DRL121 is recovered slightly better than that of JMC249 in the  $Rec^+$  host giving rise to a value of  $R=1.9$ . This result probably derives from differences in the genotype of the two phages. In experiments where genetic activity is permitted, this effect will partially mask the loss of palindrome-containing DNA molecules. Using the information from the control experiment it is therefore possible

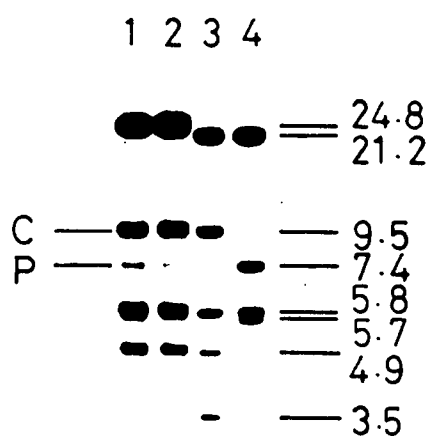


Fig. 6. DNA recovered from a *recA* host. *EcoRI* digest of supercoiled DNA of DRL110+JMC249 recovered from W3101 *recA13* strain after 10 min (lane 1) and 60 min (lane 2); and linear DNA of JMC249 (lane 3) and DRL110 (lane 4) extracted from phage particles. Bands are labelled according to their size (in kilobase pairs) and bands P and C were used to compare the yields of the two supercoiled DNAs (see Fig. 2)

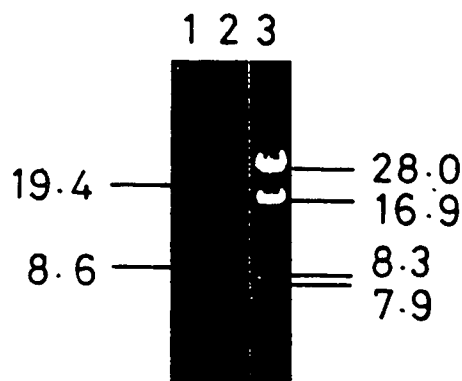


Fig. 7. DNA recovered from a *recA* host. *SmaI* digest of: linear DNA of JMC249 (lane 1) and DRL110 (lane 2) extracted from phage particles; and supercoiled DNA of DRL110+JMC249 recovered after 60 min from W3101 *recA13* strain. Bands are labelled according to their size (in kilobase pairs). The 7.9 kb band carried the palindrome

to correct the value of  $R$  obtained in the previous experiment (Fig. 4). (The corrected value of  $R$  is 0.17.)

#### Infection of *recA* cells

To observe the effects of longer periods of activity it was necessary to block DNA packaging. This was done by infecting a *recA* host. In *recA* strains *red gam* phage cannot package their DNA since it is all monomeric (see Kaiser 1971; Stahl et al. 1972 and Enquist and Skalka 1973). In this experiment we observed the ratio of DRL110 to JMC249 supercoils after 10 and 60 min of exponential growth.

The loss of palindrome-containing supercoils was

greater at 60 min than at 10 min demonstrating that the palindrome continued to confer a disadvantage to its replicon after the first 10 minutes (see Fig. 6). When compared to a simultaneous infection of the lysogenic *recBC sbcB* strain DL188 (data not shown), the 10 min infection gave a value of *R* of 0.14 and the 60 min infection a value of approximately 0.04. The last figure is only approximate due to inaccuracies in the measurement of peaks of very dissimilar size.

In order to determine whether the DNA from DRL110 present in *recA* cells after 60 min had undergone any gross rearrangement (such as deletion of the palindrome) the DNA was digested with *Sma*I. The *Sma*I digest of DRL110 includes two bands of similar size (8.3 kb and 7.9 kb) the latter of which contains the palindrome (see Fig. 7). If the palindrome was deleted from the supercoils recovered in this experiment, the smaller band would be reduced to 7.4 kb in size. This has not occurred in the vast majority of molecules since the two original bands are present in equal amounts in both DNA isolated from phage particles and from *recA* cells, and no additional bands can be detected (see Fig. 7). This is true even after hybridisation with  $^{32}\text{P}$  labelled  $\lambda$  and prolonged exposure of the film (data not shown).

## Discussion

In this paper we have shown that supercoils of a replicon carrying a palindrome are poorly recovered from *Rec*<sup>+</sup> and *recA* cells whereas they are efficiently recovered from *recBC sbcB* cells. Furthermore, this loss is dependent upon the activity of the DNA since palindrome-containing DNA is efficiently recovered from *Rec*<sup>+</sup> cells lysogenic for phage lambda.

### *An Effect of the palindrome can be seen in the absence of DNA packaging*

Our results show that the presence of a palindrome has a negative effect on the level of intracellular DNA supercoils in the first 10 minutes following infection. There is, therefore, an effect of the palindrome in the absence of DNA packaging which commences approximately half way through the latent period (see Echols 1971; Kellenberger and Edgar 1971). We have not ruled out the possibility that an additional effect may occur at the level of packaging but we have no results that require this to be the case. The result agrees with the observation that palindrome-conferred inviability occurs both in phage and plasmid replicons.

### *An effect of the palindrome can be seen in the absence of recombination*

The results from the infection of *recA* cells indicate that an effect of the palindrome occurs at the level of monomeric supercoils. This argues against the hypothesis that the inhibition of dimerisation caused by a block to recombination is the sole cause of palindrome inviability in *Rec*<sup>+</sup> cells. This hypothesis must be ruled out because the packaging of *red gam* phage is entirely dependent on dimerisation by recombination in *RecBC*<sup>+</sup> cells whereas in *RecBC*<sup>-</sup> cells recombination is not required since rolling-circle replication can occur.

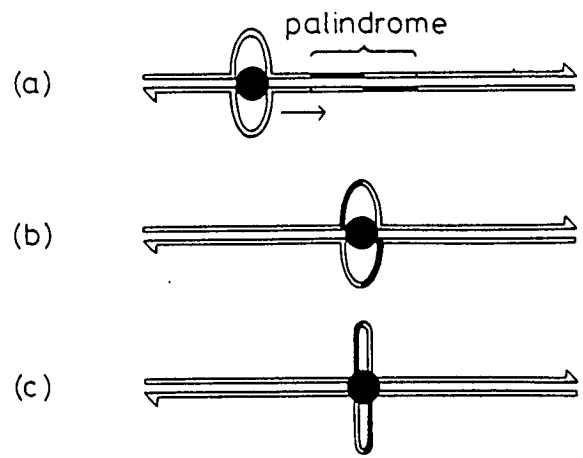


Fig. 8a-c. A model for cruciform extrusion catalysed by *RecBC* enzyme. *RecBC* enzyme migrates along the DNA unwinding the strands as it proceeds (a). When a large palindrome is reached (b) intrastrand base pairing can occur to generate a cruciform (c). This may then lead to cleavage of the four-way Holliday-like junction due to prolonged exposure to the enzyme which has been blocked in its progress along the DNA. A similar blockage might be expected to occur at real Holliday junctions formed during recombination and lead to resolution of the intermediate. Alternatively, the cruciform itself may inhibit an essential activity of the DNA (e.g. its replication)

Warren and Green (1985) have shown that the ratio of monomeric to dimeric plasmids can be increased if a palindrome is present and they suggest that this may be caused by survival disadvantage of the dimeric species rather than a change in recombination rates. This effect may also occur with the lambda DNAs observed here, but it does not explain the significant effect of the palindrome on monomeric circles in the *recA* host.

### *Loss of palindrome-containing supercoiled DNA occurs only when the DNA is active*

A comparison of infections into lysogenic and non-lysogenic bacteria has shown that the *Rec*<sup>+</sup>-dependent loss of palindromic DNA occurs only if that DNA is active. Two classes of hypotheses can be put forward to account for an activity-dependent loss. The first is simply that a palindrome interferes with an aspect of active DNA (e.g. replications or transcription). The second is that DNA activity is a prerequisite for the action of whatever is directly responsible for inviability (e.g. cleavage).

The differences observed in this work between the *Rec*<sup>+</sup> and *recBC sbcB* strains occur at the level of supercoiled DNA molecules. This raises the question of how the *recBC* and *sbcB* mutations exert this effect on active supercoiled molecules. Perhaps the unwinding activity of the *RecBC* enzyme leads to the formation of cruciform DNA (see Fig. 8) and this is linked to DNA activity in either of two ways. The first possibility is that cruciform DNA generated by *RecBC* enzyme inhibits a feature of active DNA. The second possibility is that DNA activity is a prerequisite for the action of the *RecBC* nuclease. This could be because activity creates an entry site for the enzyme. If this second explanation is correct, inviability might result from *RecBC*-dependent cleavage. The role of the *sbcB* mutation in this phenomenon remains unclear, though it appears to be less crucial than that of *recBC* (Wyman et al. 1985, D. Leach,

unpublished observations). The suggestion that RecBC enzyme catalyses cruciform extrusion is consistent with the kinetic experiments which indicated that uncatalysed cruciform extrusion might not occur frequently in vivo (Courey and Wang 1983; Gellert et al. 1983). Also, since RecBC enzyme is known to generate long single-strand loops in vitro (Taylor and Smith 1980) pairing of the arms of the palindrome may occur via a pathway independent of central symmetry (see Fig. 8). This is consistent with the observation of Warren and Green (1985) that insertions of up to 50 base pairs in length to the centre of a palindrome does not reverse its inviability. How RecBC enzyme enters the DNA is unclear since in vitro unwinding requires a double strand break in the substrate (Taylor and Smith 1985). Perhaps transient double strand breaks are utilised (e.g. upon injection or attempted initiation of rolling circle replication) or the requirement for a double strand break can be overcome in vivo.

Poor transcription of palindrome-containing DNA is unlikely to be the cause of DNA loss since complementation will occur from JMC249 which will be present in more than 85% of cells infected with DRL110 at the multiplicities used. Replication is therefore the most likely activity to be involved in the loss of supercoils reported here. Support for this view has been obtained by Shurvinton, Stahl and Stahl who have shown, in density transfer experiments, that replication is closely associated with the poor recovery of this palindrome-containing phage (personal communication).

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