dif site-specific recombination and chromosome segregation

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by

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Dedicated with love to my family

The research presented here, except where otherwise stated, is my own and original work, and has not been submitted for any other degree.

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Abbreviations

Ap	ampicillin
ATP	adenosine triphosphate
Cm	chloramphenicol
DAPI	4',6-diamidino-2-phenylindole
DNA	deoxyribonucleic acid
dNTP	2' deoxyribonucleoside
EDTA	ethylenediaminetetra-acetic acid (disodium salt)
Gm	gentamicin
IPTG	isopropyl-β-D-thiogalactopyranoside
Km	kanamycin
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
Tc	tetracycline
Tris	Tris(hydroxymethyl)aminoethane
X-gal	5-bromo-4-chloro-3-indoyl-β-D-galacto-pyranoside
min.	minutes
sec.	seconds
bp	base pairs
kb	kilobase pairs
Mb	megabase pairs
kDa	kilodaltons
OD	optical density
ORF	open reading frame
UV	ultraviolet light
X °	a temperature of X degrees centigrade
X^R	resistance to antibiotic X
X^S	sensitivity to antibiotic X

Summary

Linear chromosomes, for example those of eukaryotes, have evolved mechanisms to ensure that chromosome ends are protected from exonucleolytic attack and can be replicated completely. Circular chromosomes are not subject to these problems. However, their circularity makes homologous recombination a threat to their integrity and maintenance. Any number of homologous recombination events between linear homologous chromosomes generates linear products that have the same overall structure as their parents. In contrast, any odd number of homologous exchanges between circular chromosomes generates a fusion of the two circles. Such dimeric molecules might create difficulties in segregation at cell division, or in packaging when the circles are viral. Clearly, a unit copy replicon in dimeric form can not be normally partitioned into two daughter cells. However, multimerisation also interferes with the stable inheritance of high copy number plasmids. It is therefore not surprising to find that circular genomes have evolved mechanisms to ensure that multimers can be effectively converted to monomers. In *Escherichia coli* and related bacteria, we believe that both plasmids and the bacterial chromosome use site-specific recombination to convert multimers to monomers.

The replication terminus region of the *E. coli* chromosome encodes a locus, *dif*, that is required for normal chromosome segregation at cell division. *dif* is a substrate for site-specific recombination catalysed by the related chromosomally encoded recombinases XerC and XerD. It has been proposed that this recombination converts chromosome multimers formed by homologous recombination back to monomers in order that they can be segregated prior to cell division. Strains mutant in *dif*, *xerC*, or *xerD*, share a characteristic phenotype, containing a variable fraction of filamentous cells with aberrantly positioned and sized nucleoids. It is shown that the only DNA sequences required for wild-type

dif function in the terminus region of the chromosome are contained within 33 bp known to bind XerC and XerD, and that certain active site residues of the Xer proteins known to be involved in the catalysis of recombination are required for normal chromosome segregation. It is also shown that recombination by the *loxP*/Cre system of bacteriophage P1 will suppress the phenotype of a *dif* deletion strain when *loxP* is inserted in the terminus region. Since neither the *dif*/Xer, nor the *loxP*/Cre system caused this suppression when located in other positions on the chromosome, close to *oriC* or within *lacZ*, this reinforces the idea that site-specific recombination must occur in the terminus region in order to allow normal chromosome segregation.

Chapter 1
Introduction

1.1 Site-specific recombination

Conservative site-specific recombination is a biological process by which precise rearrangements are made to DNA molecules by breakage and rejoining at specific sequences without the degradation or synthesis of DNA. This process occurs in both prokaryotes and eukaryotes, and is catalysed by specific enzymes, known collectively as recombinases. In this recombination, a pair of specific nucleotide sequences at which recombination takes place, known as recombination sites and generally from 25-250 bp in size, are brought together through the action of proteins that recognize and bind specifically at these sites. These DNA sites are then cleaved at precise points by the recombinase enzymes, and DNA ends from different reacting sites are re-ligated to form a recombinant product. Site-specific recombination is differentiated from homologous recombination and transposition, as recombination occurs between two specific, short and often identical, sequences. The process of homologous recombination has no requirements for specific sequences, as recombination can occur between any sequences brought together by extensive homologous base-pairing mediated by RecA and similar proteins, although specific sequences are implicated in some cases in the initiation of this recombination (reviewed by Smith, 1988, 1991; West, 1992). Transposition on the other hand is not recombination between pairs of specific or homologous sequences, but rather the mobilisation of a particular sequence (the transposon) from one genetic location into another, generally unspecified, location by recombination at the ends of the transposon. Transposition is also accompanied by the synthesis of new DNA (reviewed by Mizuuchi, 1992; Haniford and Chaconas, 1992). Site-specific recombination has been reviewed by Craig, (1988), and Stark *et al.*, (1992).

1.2 The biological functions of site-specific recombination.

All natural site-specific recombination sites contain a sequence asymmetry, ensuring that during recombination the left half of one site is rejoined to the right half of the other site, and *vice versa*. Therefore, recombination between a pair of sites will result in one of three types of rearrangements, termed fusion (or integration), resolution (or excision), or inversion, depending upon the relationship of the sites on DNA before recombination. The exact results depend upon whether sites are in circular or linear DNA molecules. These three types of rearrangement are shown in Fig. 1.1. The reactions shown are topologically simple, corresponding to those detected in the *in vivo* environment in which DNA gyrase and other topoisomerases act to maintain DNA as non-catenated negatively supercoiled molecules. However, *in vitro* recombination has shown the production of topologically complex catenated and knotted products (e.g. Fig. 1.1d).

The rearrangement of DNA molecules in these ways has been implicated in many natural functions.

i. Bacteriophage integration and excision. The process of site-specific recombination was first described through studies of bacteriophage lambda (Campbell, 1962). Lambda, and many other bacteriophages use site-specific recombination to integrate the 'phage genome into the host chromosome, allowing copying by the host cell's replication machinery, as part of a dormant, or lysogenic, portion of the 'phage life-cycle. These recombination systems are complex and carefully regulated, ensuring that integration and excision of the 'phage genome into and out of the host chromosome are dependant upon the lytic/lysogenic status of the 'phage (reviewed by Landy, 1989, and Thompson and Landy, 1989).

ii. Inversion of DNA segments, controlling gene expression. A family of related recombination systems, the DNA invertase family, control the inversion of DNA

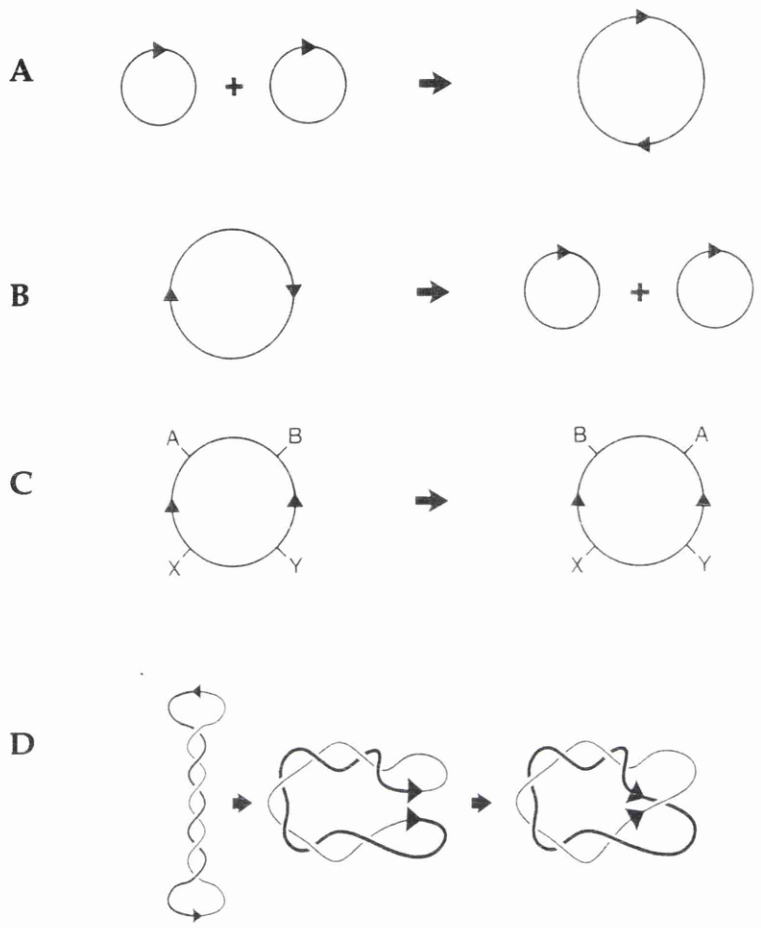


Figure 1.1. Possible genetic rearrangements to circular DNA molecules resulting from site-specific recombination. A pair of site-specific recombination sites (shown as arrowheads) in circular molecules will be in one of three relative configurations, in different molecules (A), or in the same molecule in direct (B) or inverted (C) repeat. Productive recombination between correctly aligned sites in these configuration will therefore cause fusion (integration), deletion (excision) or inversion respectively, as shown in the figure. Section D of the figure shows an example of the possible topological complexity of recombination - a topologically complex resolution reaction resulting in a multiply linked catanane. This reaction would only be detected under special conditions or in *in vitro* recombination. This figure is adapted from Craig, 1988.

segments containing genes or promoter sequences, governing the variation of the flagellar antigens in *Salmonella typhimurium* (the Hin system) and the tail fibre proteins of bacteriophages Mu (Gin), P1 (Cin) and relatives. The function of flagellar variation in *S. typhimurium* appears to be evasion of the hosts immune system, and that of bacteriophage tail fibre variation seems to be to allow changes in 'phage host range (the DNA invertase family reviewed by Glasgow *et al.*, 1989 and Johnson, 1991). The *E. coli* chromosome also encodes a similar inversion system, controlling the expression of type 1 fimbrae through inversion of the promoter region of the fimbrial sub-unit gene *fimA*. However, inversion of this DNA segment is mediated by a site-specific recombination system unrelated to the invertase family, using the two related recombinases *fimB* and *fimE* (Klemm, 1986).

iii. Resolution of transposition intermediates. Prokaryotic transposons of the Tn3 family have been shown to transpose from one DNA replicon to another by a two step replicative mechanism, the first step of which is the production of a co-integrate fusion molecule, comprising the donor and target DNA separated by two copies of the transposon. These intermediates are then resolved by site-specific recombination between copies of a recombination site in the transposon, *res*, catalysed by a transposon encoded recombinase, resolvase. Resolvase recombination has been reviewed by Sherratt, (1989), Stark *et al;*., (1989b) and Grindley, (1994).

iv. Plasmid multimer resolution. Many, and perhaps all, natural plasmids contain site-specific recombination systems that contribute to their stable inheritance by converting plasmid dimers and other multimers formed by homologous recombination back to monomers. These systems will be discussed in further detail below.

v. Plasmid amplification. The yeast 2μm circle plasmid and bacteriophage P1 may use site-specific recombination to cause amplification of their genomes. It appears that by inversion of a large segment of a circular genome, and therefore a

replication fork within this segment, replication is not terminated by a meeting of the two replication forks (Futcher, 1986; Adams *et al.*, 1992a).

1.3 Mechanisms of site-specific recombination

The strand exchange reactions of site-specific recombination are catalysed at 'core recombination sites' or 'crossover sites', usually by one recombinase enzyme. These core sites are usually comprised of two binding sequences for the acting recombinase enzyme(s) in inverted repeat, separated by a central, or overlap region, containing the specific positions at which these enzymes catalyse strand cleavage and rejoining (Fig. 1.2). In many systems, although the sequence of the central or overlap region can be altered without greatly effecting recombination, these changes must exist in both recombining partners, as the overlap region appears to be the only position in which homology between sites is required for efficient recombination (Kitts and Nash, 1987; Craig, 1988; Stark *et al.*, 1989). Results suggest that overlap homology may not be as stringently required in the Xer recombination system (Summers, 1989; McCulloch, 1992, 1994).

In most systems, a core site is not a sufficient substrate for recombination. Full recombination sites often include 'accessory sequences', additional protein binding sequences usually adjacent to the core site; recombination requires the binding of proteins to these sequences, either further binding of the recombinase enzyme(s), or additional 'accessory proteins'. These accessory sequences and proteins are not intimately involved in the catalysis of strand exchange, but are implicated in bringing core sites close together in the required configuration for active recombination. This 'coming together' of recombination sites in an active configuration is termed synapsis, or synapse formation. Accessory sequences and accessory proteins appear to mediate synapsis through protein-DNA and

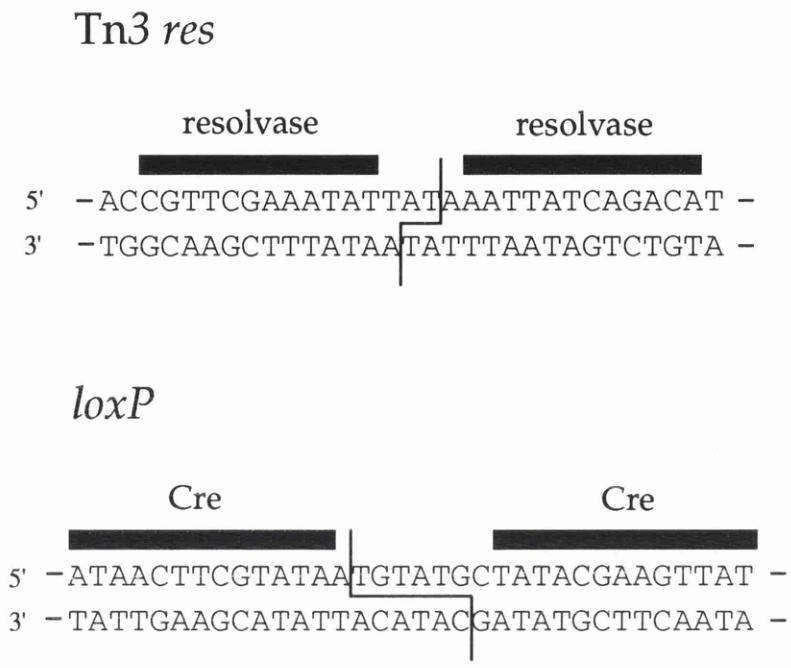


Figure 1.2. A comparison of the core recombination sites of the Tn3 *res*/resolvase and bacteriophage P1 *loxP*/Cre recombination systems. Tn3 resolvase and Cre are members of the resolvase/DNA invertase and integrase families of site-specific recombinases respectively. This diagram shows the characteristic differences between core recombination sites acted upon by members of the two families. The nucleotide sequence of each site is shown. Staggered black lines show the points at which strands are broken in each reaction, defining the 'overlap region' between these two points. In integrase family recombination, these two strand breakages are not made simultaneously. Thin black boxes show the recognition sequences to which the stated recombinase enzyme binds, flanking the points of strand exchange. The length of the overlap region varies from 6-8 bp between members of the integrase family.

protein-protein interactions. This view is supported by the known DNA binding and bending activity of well characterised accessory proteins, such as IHF (Freundlich *et al.*, 1992; Landy, 1993), Fis (Finkel and Johnson, 1992) and ArgR (Charlier *et al.*, 1992; Burke *et al.*, 1994).

The mechanisms by which recombinase enzymes catalyse recombination at core sites has been extensively studied through the reconstitution of recombination reactions *in vitro*, using purified or partially purified recombinase enzymes and specific DNA substrates in simple buffers, first achieved by Nash, (1975). No high energy co-factors, such as ATP, are required for site-specific recombination. Although there are many similarities in the mechanisms by which all recombination systems seem to function, those studied do have many different characteristics. Each system characterised has been found to fall into one of two apparently unrelated families that will be discussed separately. These families are not differentiated by means of the complexity of regulation, or function of their recombination, but simply on the use of what appear to be different evolutionarily conserved groups of recombinase proteins and two distinct recombination mechanisms. Interestingly, Lenich and Glasgow (1994) recently described a possible recombinase enzyme without homology to either well characterised family, but with homology to a group of transposases. However, it still remains for the recombinase activity of this protein to be demonstrated unquestionably.

1.4 The resolvase/invertase family of site-specific recombinases

Recombination by the resolvase enzymes of transposons Tn*3*, and γδ, and the DNA invertases Gin and Hin has been extensively studied *in vitro* and appears to have a common catalytic mechanism (Reed, 1981; Kahmann *et al.*, 1985; Bruist *et al.*, 1987; Stark *et al.*, 1992; Grindley, 1994). The amino acid sequence and the size

of these enzymes is conserved, and is shared with many other recombinase enzymes, the resolvase enzymes of transposons including Tn*21*, Tn*552*, Tn*917*, Tn*501*, the invertases Pin and Cin, the plasmid multimer resolvases from R46 and RP4, a sporulation specific excision enzyme from *Bacillus subtillis* and others (Dodd and Bennett, 1987; Hatfull and Grindley, 1988; Glasgow, 1989; Sherratt, 1989; Rowland and Dyke, 1989, Gerlitz *et al.*, 1990, Sato *et al.*, 1990). It would be expected that these other resolvase/DNA invertase proteins also share the same catalytic mechanism. These proteins are generally small (≈20 kD, ≈185 amino acids), and are sub-divided into two domains, a large amino terminal catalytic domain, and a smaller, carboxy terminal domain believed to bind DNA using a helix-turn-helix motif (Abdel-Meguid *et al.*, 1984; Grindley, 1994).

The core recombination sites and the proposed mechanisms for catalysis of the resolvase/DNA invertase family members are similar, the points of strand exchange on the two DNA strands being separated by a two base pair overlap or central region. It is believed that strand exchange proceeds by an initial double strand cleavage, with a specific conserved serine residue of the recombinase proteins acting as nucleophiles (corresponding to S10 of Tn*3* resolvase), producing recessed 5′ ends bound to recombinase by a phosphoserine linkage, and protruding 3′-OH ends. It is then proposed that recombinant partners are brought together by a simple 180° right handed rotation of one pair of DNA ends and recombinase proteins relative to the other pair. This would then allow each 3′-OH group to attack a phosphoserine bond causing resealing of the DNA and release of the recombinase. These proposed events are shown in Fig. 1.3. This double strand cleavage and rotation model is supported by biochemical data detecting the proposed reaction intermediates, cut in all four strands and covalently linked to DNA by a phosphoserine linkage and by topological studies of recombination (Reed and Grindley, 1981; Hatfull and Grindley, 1986; Klippel *et al.*, 1988a; Stark *et al.*, 1989a, Stark and Boocock, 1994).

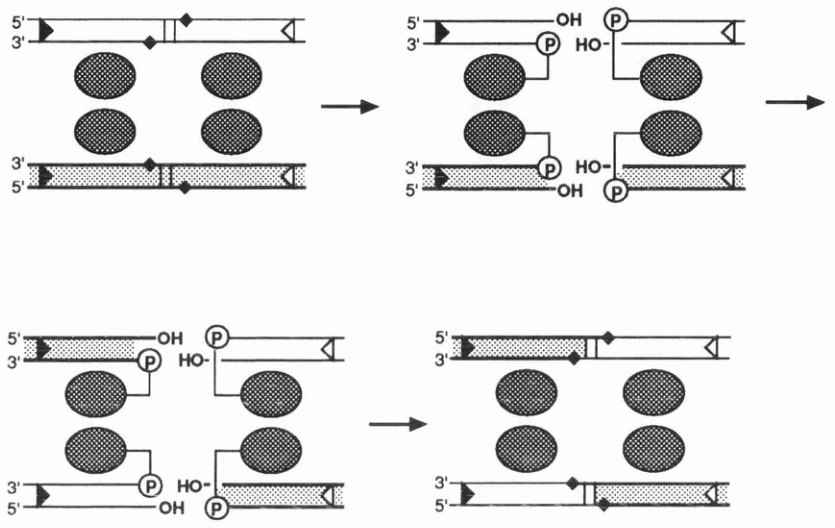


Figure 1.3. Catalysis of site-specific recombination between two core recombination sites by enzymes of the resolvase/DNA invertase family. Protein subunits are represented by shaded ovals; the outside ends of the recombinase recognition sequences by inverted black and white arrowheads; the two base pairs in the overlap region by vertical lines; and the phosphates that are attacked by the recombinase by black diamonds. DNA strands from different recombining partners are differentiated as thick and thin lines, and by shading. Recombination is believed to proceed as described in the main text, by simultaneous cleavage of both strands, and rejoining after 180° right handed rotation of one pair of half-sites relative to the other. This diagram was adapted from Stark *et al.*, (1992).

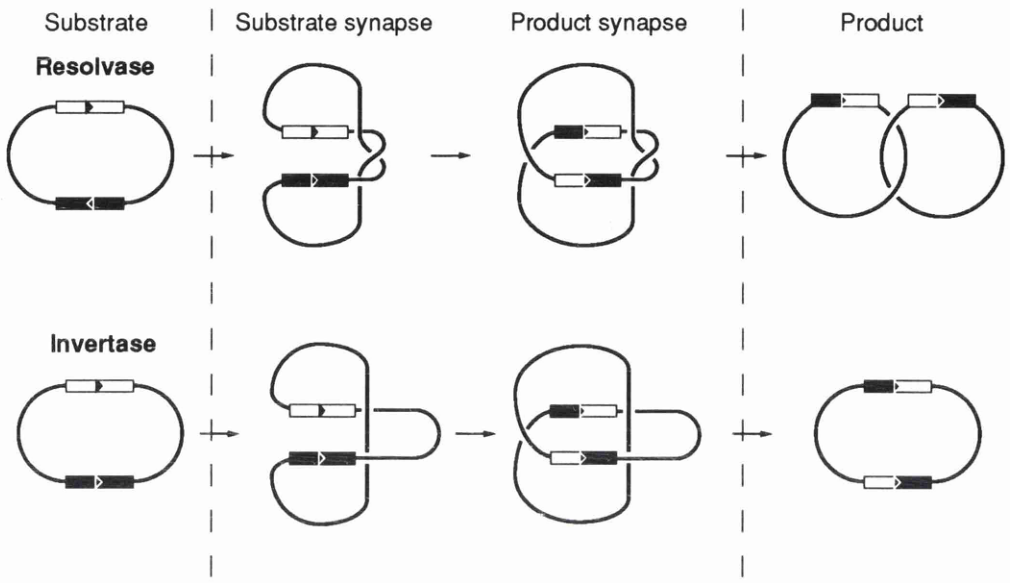


Figure 1.4. The topology of reactions catalysed by resolvases and DNA invertases. This figure shows proposed topological pathways for resolvase and DNA invertase recombination between sites in a circular molecule. Core recombination sites are shown as boxes containing arrows, in each case differentiated as one black and one white site in the substrate. For both reaction pathways, the events occurring at the core site are the same. This figure is adapted from Stark *et al.*, (1992).

The full recombination sequence requirements of both the resolvase and invertase enzyme groups each have a characteristic organisation, implicated in the selectivity of recombination at these sites; resolvases will only catalyse recombination between sites (*res* sites) in direct repeat within the same DNA molecule (Reed, 1981; Fig. 1.1.b, Fig. 1.4), whereas invertases will only catalyse recombination between sites in inverted repeat (Kahmann *et al.*, 1985; Fig. 1.1.c, Fig. 1.4). *res* sites are comprised of the core recombination site, known as sub-site I, and two other similar sequences that also bind resolvase, but do not undergo strand exchange reactions, sub-sites II and III (Stark *et al.*, 1989b; Grindley, 1994). Although each site required for invertase recombination is simply a core site without accessory sequences, recombination between two sites also requires a recombination enhancer site, *sis*, that binds the FIS protein and may act at a variable distance from the two core sites (Kahmann, 1985; Glasgow, 1989; Finkel and Johnson, 1992). The mechanisms by which these accessory sequences and accessory proteins enforce selectivity upon recombination will be discussed later.

1.5 The λ integrase family of site-specific recombinases

Since the characterisation of the integrase recombination system of bacteriophage λ, many other recombination systems have been discovered that have a similar recombination mechanism, and use enzymes with homology to the λ integrase recombinase. The best studied members of this enzyme family, with which recombination has been reconstituted *in vitro*, are λ integrase itself, the Cre protein from 'phage P1, FLP from the 2μm circle plasmid of *Saccharomyces cerevisiae* and the XerC and XerD recombinases of *E. coli* (Cox, 1989; Landy, 1989; Hoess and Abremski, 1990; Sherratt *et al.*, 1995). However, there are many other recombination systems using enzymes with homology to this integrase family, that would be expected to share other properties with these well characterised

systems, including several other 'phage and transposon integration systems, the fimbrial inversion systems of *E. coli* and *Proteus mirabilis*, the transposon cointegrate resolution system of Tn*4430*, and several plasmid multimer resolution systems (Argos *et al.*, 1986; Klemm, 1986; Lane *et al.*, 1986, O' Connor *et al.*, 1986; Bastos and Murphy, 1988; Mahillon and Lereclus, 1988; Krause and Guiney, 1991; Bahrani and Mobley, 1994). When compared to the resolvase/DNA invertase family of recombinases, the λ integrase family are less conserved in amino acid sequence and in size, and appear to be used in systems from more divergent origins, with more divergent functions. Argos *et al.*, (1986), in defining the family, highlighted that sequence conservation was generally restricted to two specific regions of the proteins. Within these two domains are found the four completely conserved residues of the integrase family, believed to play important roles in catalysis (Argos *et al.*, 1986; Abremski and Hoess, 1992). The possible roles of these 'invariant tetrad' residues is discussed further in Chapter 6.

The core recombination sites at which recombination is catalysed by integrase family enzymes do not have the exact conservation of structure found with the resolvase/DNA invertase family; the two positions at which DNA strands are cleaved are separated by overlap regions of various lengths in different systems, generally from 6 to 8 bp. These positions of strand cleavage are staggered differently than those with the resolvase/DNA invertase family (Fig. 1.2). Whereas in resolvase recombination it is believed that double stranded cleavage causes the production of protruding 3'-OH ends, it appears that integrase recombination does not proceed by a simultaneous cleavage of all four strands of two recombining duplexes, but that each pair of strand exchanges is performed independently, recombination proceeding via a Holliday junction intermediate. There is strong evidence for this, as, with each well characterised integrase system, Holliday structures have been isolated during recombination, usually under abnormal conditions or using mutant proteins or sites (Hoess *et al.*, 1987; Nunes-Duby *et al.*, 1987; Jayaram *et al.*, 1988; Kitts and Nash, 1988a; Meyer-

Leon *et al.*, 1988, 1990; McCulloch *et al.*, 1994). It has been shown that in each system, a particular set of strands are normally exchanged first, the 'top' strands (Hoess *et al.*, 1987; Kitts and Nash, 1988b; Landy, 1989). For resolution of this Holliday structure, the junction must branch migrate through the overlap sequence to the point at which the second strand exchanges occur. It appears that it is this branch migration step that imposes a requirement for homology between recombining partners within the overlap sequence (Nunes-Düby *et al.*, 1987; Kitts and Nash, 1987). After branch migration, the second set of strands are exchanged to give recombinant products. The proposed strand exchange mechanism for integrase recombination is shown in Fig. 1.5.

The complexity of complete recombination sites used in integrase family recombination, and similarly the complexity of regulation of recombination reactions performed, is very diverse. The integration system of bacteriophage λ is a complex and highly regulated recombination system, utilising four different proteins and large non-identical recombination sites. The large, complex (≈240 bp) *attP* site of the 'phage genome can integrate into a small simple (≈30 bp) target site on the *E. coli* chromosome, *attB*, leaving two complex hybrid sites at either end of the 'phage genome, *attL*, and *attR*. These two hybrid sites can react to excise the 'phage genome, recreating *attP* and *attB*. Recombination is carefully regulated to coincide with changes in the infective status of the bacteriophage. In stark contrast to this are the *loxP*/Cre system of bacteriophage P1 and the *FRT*/FLP system of the yeast 2μm plasmid. They require no accessory sequences beyond a core recombination site, and appear to show no selectivity *in vitro* for sites in a particular configuration. They will perform efficient fusion, resolution or inversion reactions using sites on supercoiled, relaxed, or even linear substrates.

These differences in site complexity also require differences in the mechanism by which sites are correctly aligned for recombination. In order to determine the left and right of a site, some sequence asymmetry is required.

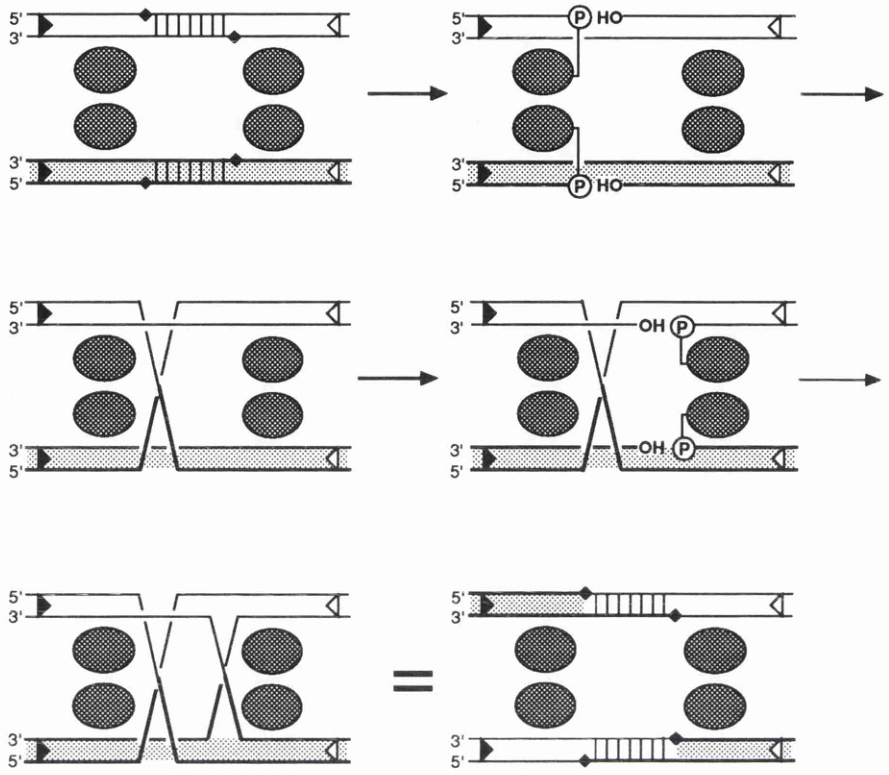


Figure 1.5. Catalysis of site-specific recombination between two core recombination sites by enzymes of the integrase family. Protein subunits are represented by shaded ovals; the outside ends of the recombinase recognition sequences by inverted black and white arrowheads; the base pairs in the overlap region by vertical lines (the size of the overlap region varies from 6-8 bp within the integrase family); and the phosphates that are attacked by the recombinase by black diamonds. DNA strands from different recombining partners are differentiated as thick and thin lines, and by shading. Recombination is believed to proceed as described in the main text, by sequential pairs of strand exchanges, progressing through a Holliday junction intermediate. The figure is not intended to imply that two four-way junctions are present simultaneously. Protein subunits are shown to cleave the DNA strand to which they are bound, termed *cis* cleavage. Although cleavage in *trans* has been observed experimentally with some members of the integrase family, the question of *cis* or *trans* cleavage is currently unresolved (Lee *et al.*, 1994; Nunes-Düby *et al.*, 1994). This Figure is adapted from Stark *et al.*, (1992).

Whereas in 'phage λ *att* sites (as with Tn3 *res*) this asymmetry is supplied by the accessory sequences flanking the core site, in *loxP* and *FRT* sites it is asymmetry within the overlap sequence that is important, and in the *dif* site of the *E. coli* chromosome, acted upon by the two recombinases XerC and XerD, it appears to be the binding of different recombinase proteins to different halves of the core site that mediates site alignment (Hoess *et al.*, 1986; Senecoff and Cox, 1986; Landy, 1989; Blakely *et al.*, 1993).

1.6 Recombination selectivity

In order to fulfil their biological functions, many recombination systems will only function when sites are configured on DNA in a particular way. As described earlier, recombination between a pair of sites will result in one of three types of rearrangements, termed fusion (or integration), resolution (or excision), or inversion, depending upon the relationship of the sites on DNA before recombination (Fig. 1.1). Many recombination systems include mechanisms to ensure that recombination will only be catalysed between sites in a specific relative configuration, and therefore that only one of these classes of rearrangement will occur. There is good evidence from a number of systems that this selectivity is enforced by accessory sequences and accessory proteins (Klippel *et al.*, 1988b; Landy, 1989; Summers, 1989; Crisona *et al.*, 1994; Stark and Boocock, 1995), and indeed, since some core recombination sites alone are able to recombine non-selectively, it might be suggested that the only explanation for the use of accessory sequences and proteins is the enforcement of selectivity.

Tn3 resolvase will only recombine sites in direct repeat within the same DNA molecule, causing resolution into two daughter molecules (Reed, 1981; Kitts *et al.*, 1983). Topological investigation of this reaction *in vitro* showed that the product molecules were exclusively formed as a singly linked, -2 catenane

(Reed, 1981; Krasnow and Cozzarelli, 1983; Wasserman and Cozzarelli, 1985). This showed that this reaction had not simply resolution selectivity, but was topologically selective. Further investigation of the DNA linkage change of the reaction and the topology of minor reaction products support a model for resolvase recombination in which site synapsis must trap exactly three interdomainal nodes between the core recombination sites (Wasserman and Cozzarelli, 1985; Wasserman *et al.*, 1985; Stark *et al.*, 1989a; Stark and Boocock, 1995). It is proposed that this requirement for the wrapping of the substrate DNA to produce three nodes ensures that the production of the correct recombination synapse topology, and the production of recombinants through this 'topological filter', is only energetically favourable when sites are in direct repeat within the same molecule (Stark and Boocock, 1995). Fig. 1.6a shows this proposed topology of resolvase recombination, and Fig. 1.6b the topological consequences of recombination by this pathway between two sites in inverted repeat or on separate molecules. It is believed that a similar mechanism of selectivity applies to Gin and Hin mediated inversion recombination, but that two interdomainal nodes are trapped between sites (Hatfull and Grindley, 1988; Kanaar *et al.*, 1988, Stark and Boocock, 1995; Fig. 1.4). Recent results investigating the topology of Xer recombination at *psi* and *cer* (Summers and Sherratt, 1984, Cornet *et al.*, 1994, Sherratt *et al.*, 1995) have suggest that these systems, unrelated to the resolvase/DNA invertase systems may use a mechanism of resolution selectivity very similar to that proposed for *res* (S. Colloms and D. Sherratt, unpublished data). These experiments are discussed further in Chapter 5.

1.7 The Xer site-specific recombination system

The Xer recombination system was identified through the role that it plays in the stable inheritance of the natural high copy number plasmid ColE1 (Summers and

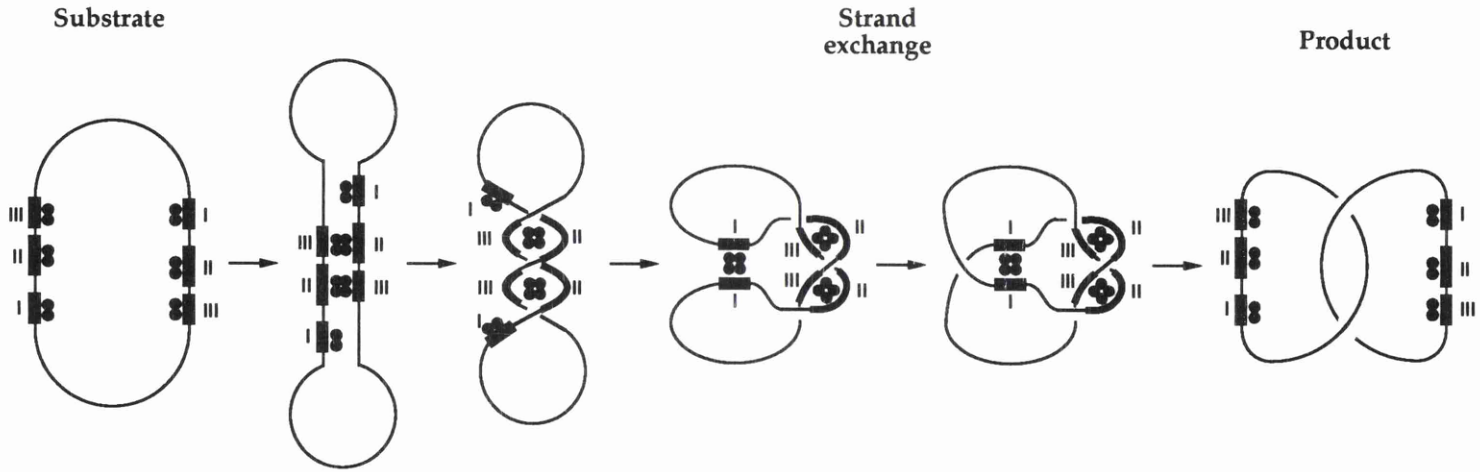


Figure 1.6a. Model for *res*/resolvase topological selectivity - the topological effects of recombination between two directly repeated *res* sites. The three sub-sites are identified by roman numerals. It is proposed that a requirement for the production of the substrate synapse in the figure ensures that recombination is only energetically favourable between sites in the configuration shown. Strand exchange proceeds with a 180° right handed rotation. This figure is adapted from Stark and Boocock, (1995).

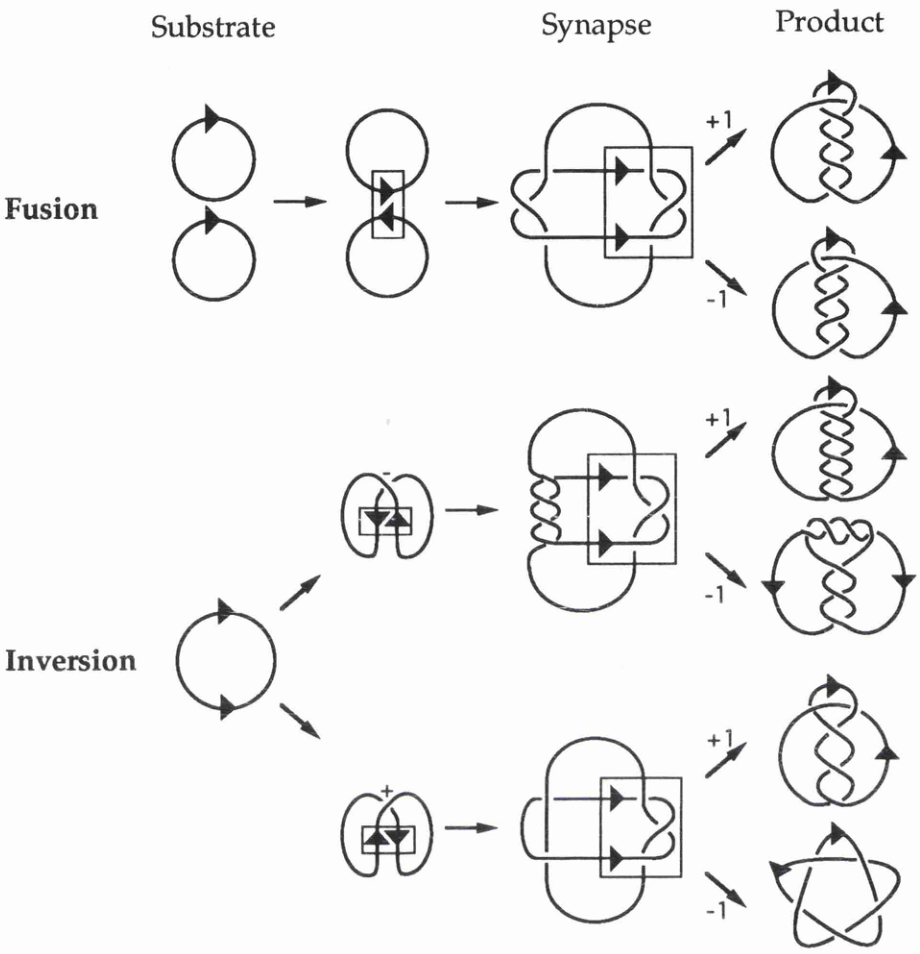


Figure 1.6b. Model for *res*/resolvase topological selectivity - the topological effects of recombination between sites on separate molecules or in inverted repeat. This figure shows the possible topological consequences of fusion and inversion reactions through the proposed synapse topology required for resolvase recombination (boxed in the figure). In each case, the effect of both right handed (+1) and left handed (-1) rotation at strand exchange is shown. It is proposed that the formation of the intermediates and products shown is energetically unfavourable, explaining why these reactions are not detected experimentally. This figure is adapted from Stark and Boocock, (1995).

Sherratt, 1984). Evidence suggests that ColE1, and related high copy number natural plasmids are randomly segregated between daughter cells at host cell division (Durkacz and Sherratt, 1973; Williams and Thomas, 1992). In a theoretical plasmid population with a copy number at cell division of n, if each plasmid copy were to segregate independently and randomly at cell division, the probability of producing a plasmid free daughter cell would be $2^{(1-n)}$. However, anything that effects the status of plasmids as independent units at segregation might reduce the effective copy number, and therefore effect plasmid stability. Homologous recombination between plasmid copies generates plasmid multimers, and since mechanisms regulating the plasmid copy number in a cell are sensitive to the actual number of copies of the plasmid's sequences in a cell, not the number of separate plasmid molecules, this multimerisation causes a drop in the number of independently segregating plasmid units in a cell, and a drop in plasmid stability (Summers and Sherratt, 1984). Once a plasmid dimer forms within a cell, this problem appears to be aggravated, as a plasmid dimer is twice as likely to be replicated as a monomer, because it contains two origins, and runaway proliferation of dimers can occur (Summers *et al.*, 1993). It was demonstrated that ColE1 encodes a locus, *cer*, that undergoes site-specific recombination, causing multimer resolution and increased stability (Summers and Sherratt, 1984).

The *cer* site of ColE1 is approximately 250 bp in size, and is the only ColE1 sequence required for site-specific recombination (Summers and Sherratt, 1984, 1988). Recombination shows strong resolution selectivity: recombination is only performed efficiently between copies of the site directly repeated within the same molecule. The site consists of a crossover region (the core recombination site) of approximately 30 bp, at one end of the sequence, and approximately 220 bp of accessory sequences (Summers *et al.*, 1985; Summers and Sherratt, 1988). Many other natural plasmids and transposons encode recombination systems comprising a recombination site and its cognate recombinase, for example the

rsfF/D protein system of the F plasmid (Lane *et al.*, 1986), *loxP*/Cre of bacteriophage P1 (Hoess and Abremski, 1990), or the *res*/resolvase systems of Tn3, Tn*21*, γδ etc. (Grindley, 1994). However, the small size of *cer* suggested that ColE1 did not encode its own recombinase, but was a substrate for a protein or proteins encoded on the host chromosome (Summers and Sherratt, 1984, Stirling *et al.*, 1988a). Four proteins from *E. coli* have been identified that are required for recombination at *cer* in this host, the Xer proteins, encoded by four unlinked chromosomal genes (Stirling *et al.*, 1988a, b; Stirling *et al.*, 1989, Colloms *et al.*, 1990; Blakely *et al.*, 1993).

1.8 The Xer proteins

XerA/ArgR. The *E. coli* arginine repressor is required for recombination at *cer* (Stirling *et al.*, 1988b). This protein acts in the control of the *E. coli* arginine biosynthetic genes through DNA binding to an 'ARG box' recognition sequence (Charlier *et al.*, 1992) that is also present in the accessory sequences of *cer*, separated by approximately 100 bp from the crossover region . The ArgR protein is found to bind to this sequence of *cer*, both *in vivo* and *in vitro*, in an arginine dependent fashion (Stirling *et al.*, 1988b). It appears that ArgR plays the role of an accessory protein in *cer* recombination. It appears not to be involved in the catalysis of strand exchange, but instead is implicated in enforcing recombination selectivity at *cer* through involvement in the presumptive *cer* synaptic complex - an ordered nucleoprotein complex that may enforce topological selectivity upon *cer* recombination (Summers, 1989, Burke *et al.*, 1994)

XerB/PepA. PepA, the *E. coli* aminopeptidase A protein is also required as an accessory protein in recombination at *cer* (Stirling *et al.*, 1989; Summers, 1989). As investigations have failed to detect any interaction between PepA and either *cer*

DNA or the other Xer proteins, or any requirement for its peptidase activity in *cer* recombination (Stirling *et al.*, 1989; McCulloch *et al.*, 1994b), the role of PepA as an accessory protein is very unclear. Recent results however, have shown binding of PepA to a DNA sequence making up part of a promoter for the *carAB* operon (D. Charlier, N. Glansdorff and D. Sherratt personal communication). Although this sequence does not occur in *cer*, interestingly, the control sequences of this operon are also bound by ArgR.

The Xer recombinases, XerC and XerD. Two recombinase enzymes, XerC (Colloms *et al.*, 1990) and XerD (Blakely *et al.*, 1993), are required for recombination at *cer*, and several other recombination sites (see below). Both proteins are members of the lambda integrase family of recombinases, are 298 amino acids in size, and share 37% identity (60% similarity) in sequence (Figs. 1.7 and 6.1). Each recombinase is believed to bind to recognition sequences within the crossover region of *cer* (and other related sites), XerC binding to one side, the left, of the spacer, or overlap region, and XerD to the other side, the right (Fig. 1.8; Blakely *et al.*, 1993). The chromosomal genes *xerC* and *xerD* each appear to be encoded within an operon, located at 85.7 and 62.4 minutes respectively on the genetic linkage map of the *E. coli* chromosome (Fig. 1.9; Bachmann, 1990). *xerC* appears to be co-expressed with *dapF* (encoding diaminopimelate epimerase), and two genes of unknown function *orf235* and *orf238* (Colloms *et al.*, 1990). *xerD* shares an operon with *recJ* and *dsbC* encoding the RecJ exonuclease and a protein actively involved in the formation of di-sulphide bridges respectively (Blakely *et al.*, 1993; Missiakas *et al.*, 1994). As yet, no functional significance has been suggested for these locations of the *xerC* and *xerD* genes, although it appears that *xerC* is closely linked to *dapF* in several related bacteria (G. Blakely, personal communication).

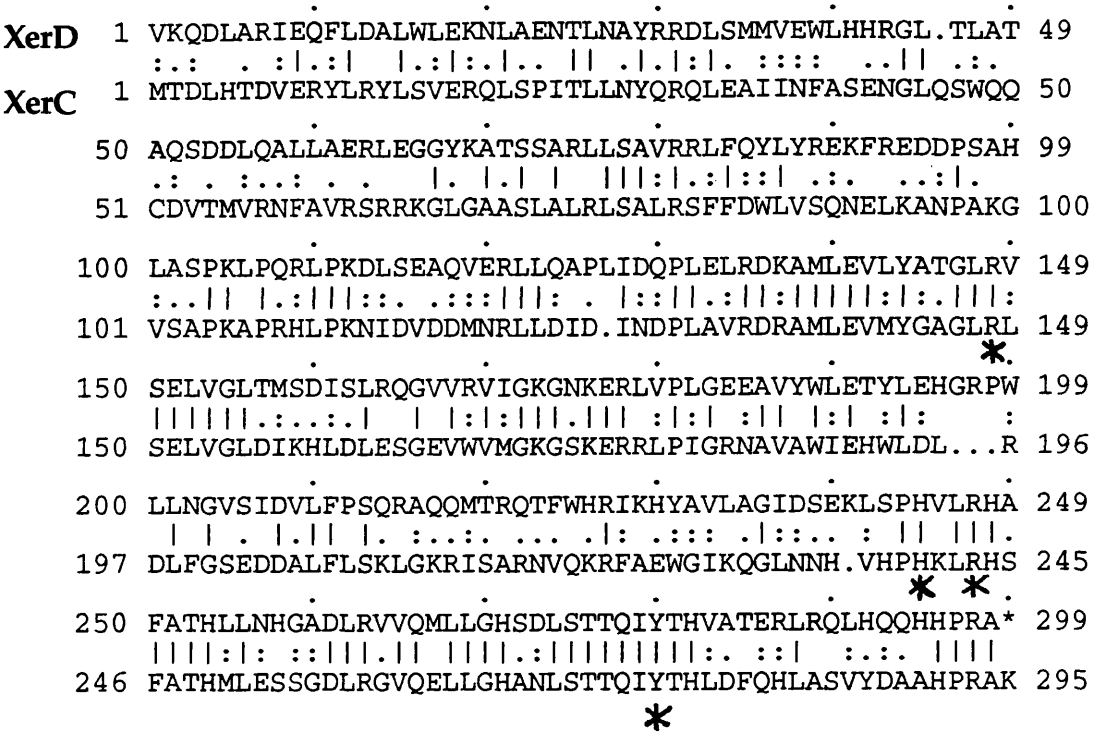


Figure 1.7. Alignment of the amino acid sequences of the lambda integrase family recombinases XerC and XerD. This figure shows the homology of amino acid sequence between XerC and XerD. Similarity is greatest within the two identified domains conserved between members of the integrase family of site-specific recombinases, approximately amino acids 145-160 and 240-280 (Fig. 6.1; Argos *et al.*, 1986; Abremski and Hoess, 1992). Within these two domains can be seen the invariant tetrad, two arginines, a histidine and the active site nucleophile tyrosine, four residues present in all members of the family, highlighted with asterisks below them.

1.9 *dif* and other Xer recombination sites

Since the discovery of *cer*, several other recombination sites from a variety of sources have been identified, with homology to *cer*, that also require the Xer proteins for activity. *cer*-like sites have been identified on many other natural high copy number plasmids related to ColE1, CloDF13 (Hakkaart *et al.*, 1984), ColK (Summers *et al.*, 1985), pMB1, (Greene *et al.*, 1981), ColA (Morlon *et al.*, 1988), and ColN (Kolot *et al.*, 1990). *cer* and these related sites appear to share a common site organisation, the requirement for all four Xer proteins and strong resolution selectivity. Sites have also been discovered from more diverse origins, with homology to *cer* limited to the crossover region, requiring only some of the Xer proteins for recombination. For example the *psi* site of plasmid pSC101 has homology to the *cer* crossover region but non-homologous accessory sequences, and requires only XerC, XerD and PepA for recombination *in vitro* (Cornet *et al.*, 1994; S. Colloms personal communication). The core recombination sites (or crossover regions) of *cer* and several other Xer recombination sites are shown in Fig. 1.8.

None of these sites give clues as to why *E. coli* should encode the Xer recombinases. However, the discovery of XerC led to the identification of *dif*, an Xer site-specific recombination site on the *E. coli* chromosome, situated in the replication terminus region (Fig. 1.9; Blakely *et al.*, 1991; Clerget, 1991; Kuempel *et al.*, 1991). The *dif* locus contains a sequence of approximately 30 bp with homology to the *cer* crossover region that is a sufficient substrate for recombination when cloned into a high copy number plasmid (Blakely *et al.*, 1991; Kuempel *et al.*, 1991). This plasmid recombination requires XerC and XerD, but not ArgR or PepA, and shows no recombination selectivity (Blakely *et al.*, 1991; Blakely *et al.*, 1993). Microscopic observation of strains with mutations in *xerC* or deletions of the chromosomal *dif* site show that both mutations cause similar morphological phenotypes. Some cells in cultures of these strains do not

left arm XerC binding	central region	right arm XerD binding	
GCGGTGCGTACAA	TTAAGGGA	TTATGGTAAAT	ColE1 (*cer*)
GCGGTGCGTACAA	TAAGGGA	TTATGGTAAAT	ColN
GCGGTGCGTACAA	CGGATG	TTATGGTAAAT	ColA
GCGGTGCGCGCAA	GATCCA	TTATGTTAAAC	pSC101
GCGGTACCGATAA	GGGATG	TTATGGTAAAT	CloDF13 (*parB*)
GCGGTGCGTACAA	TTGGGATG	TTATGGTAAAT	type I hybrid
GCGGTGCGTACAA	GGGATG	TTATGGTAAAT	type II hybrid
TTGGTGCGCATAA	TGTATA	TTATGTTAAAT	*dif*
TTGGTGCGCATAA	TTTGTATA	TTATGTTAAAT	*dif*-8

Figure 1.8. Xer core recombination sites. This figure shows an alignment of the nucleotide sequences of the core recombination sites (or crossover sites) of nine recombination sites at which XerC and XerD are believed to catalyse recombination. 'pSC101' refers to the *psi* site of this plasmid; other plasmid names refer to their *cer*-like sites. The type I and type II hybrid sites, and the *dif*-8 site were artificially created in experiments (Summers, 1989; Blakely *et al.*, 1993). *dif* and the other natural sites are described in the main text. As shown, although the XerC and XerD binding sequences of these sites are highly conserved, both the sequence and the length of the central region are variable. This figure is adapted from Roberts, (1994).

undergo normal cell division, forming long filaments with abnormally sized and positioned nucleoids, whereas most are morphologically normal. This phenotype bears some similarity to that of strains with mutations in genes implicated in the segregation of replicated chromosomes to daughter cells. Therefore, it was proposed that the function of the *dif*/Xer recombination system is to resolve chromosome multimers, formed by homologous recombination between sister chromosomes, prior to cell division (Blakely *et al.*, 1991; Clerget, 1991; Kuempel *et al.*, 1991).

1.10 Replication and partition of the *E. coli* chromosome

The circular *E. coli* chromosome, approximately 4,700 kb in length, is replicated bi-directionally. Two replication forks initiated at a specific chromosomal origin of replication, *oriC*, proceed in both directions and meet in the replication terminus region (where *dif* is naturally located, Fig 1.9; Kuempel *et al.*, 1991). This terminus region encodes a replication termination system that ensures that both replication forks meet in this region of the chromosome (discussed in detail in Chapter 7). However, this, and all other functions encoded in the terminus are non-essential, as shown by the isolation of a viable strain containing a 340 kb deletion of the entire terminus region, over 7% of the genome (Henson and Kuempel, 1985). Although the terminus contains a lower concentration of characterised genes than the rest of the chromosome, it appears that this may be due to the non-essential nature of terminus encoded functions, rather than an absence of actual coding sequences (Moir *et al.*, 1992).

Once chromosome replication is completed, before cell division can proceed, two related processes must occur. The two replicated chromosomes must be physically separated from each other, to give two independent entities, and these must then be moved apart to allow septum formation. Genes

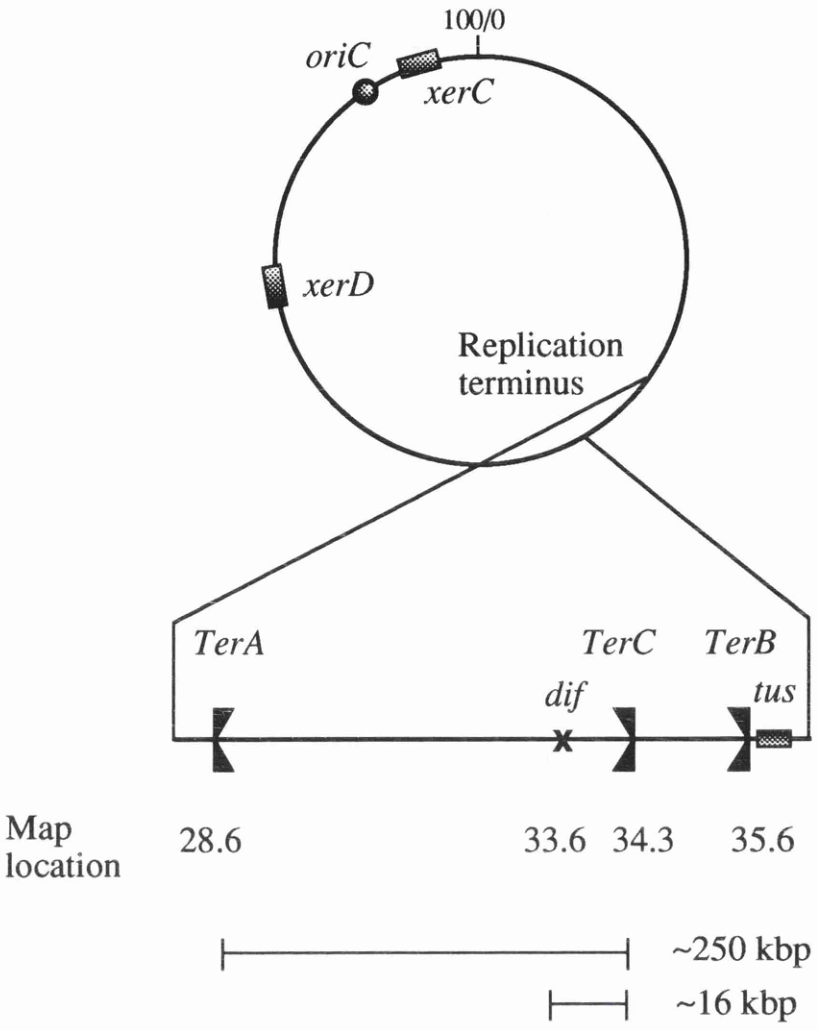


Figure 1.9. The *E. coli* chromosome - the Xer recombination system and the replication terminus region. This representation of the *E. coli* chromosome shows *xerC*, *xerD* and the position of *dif* in the replication terminus region, relative to the position 0 minutes and *oriC*. Positions in minutes refer to the genetic linkage map of Bachmann, (1990). Additional map positions are *xerC* - 85.7 minutes and *xerD* - 62.4 minutes. Three of the six identified chromosomal *Ter* sites are shown, represented such that replication forks approaching from the flat side are not affected (eg. *TerC* halts replication forks travelling from left to right).

implicated in these processes fall into three different categories, each displaying subtly different morphological phenotypes: those involved in Xer site-specific recombination, that will be discussed in greater detail in Chapter 3, those implicated in the decatenation of chromosomes (topoisomerases), and those believed to act in the physical movement of chromosomes.

Decatenation of chromosomes. How the last sequences of chromosomal DNA are replicated at the termination of replication is unclear (Løbner-Olesen and Kuempel, 1992; Hiraga, 1993). However, regardless of whether the final stretches of DNA are replicated before chromosomes are separated, the helical nature of DNA results in interlinking of the two daughter molecules, requiring decatenation. *E. coli* encodes two type II topoisomerase enzymes (capable of decatenating double stranded DNA), topoisomerase II (DNA gyrase) and topoisomerase IV. Both are essential for viability, and the investigation of strains carrying conditional lethal mutations in either protein shows the widespread formation of filamentous cells and the aberrant partition of nucleoids under non-permissive conditions (Steck and Drlica, 1984; Kato *et al.*, 1990). Recent work suggests that it is topoisomerase IV that plays a primary role in chromosomal decatenation (Adams *et al.*, 1992b).

Positioning of replicated chromosomes. Once physically independent, the two replicated chromosome copies are rapidly moved apart to positions of the cell, one quarter, and three quarters along its length. This process of nucleoid movement appears to be an active one, requiring fresh protein synthesis after chromosome replication is completed (Hiraga *et al.*, 1990; Begg and Donachie, 1991; Hiraga, 1993). Although this process is very poorly understood, screens for mutants producing a high frequency of anucleate cells have allowed the isolation of two mutants defective in this process. One mutation was found to map in *tolC*, encoding an outer membrane protein, and the other to define a new gene *mukB*.

This latter gene encodes a 177 kDa protein, MukB, able to bind to DNA, ATP and GTP and with structural and sequence homologies to several force generating enzymes from eukaryotes, e.g.. myosin heavy chain and dynamin D100 (Niki, *et al.*, 1991, 1992). The phenotypes caused by mutations in either *tolC* or *mukB* are characterised by the widespread production of anucleate cells and the incorrect positioning of chromosomal material (Hiraga *et al.*, 1989; Niki *et al.*, 1991).

1.11 The biological function of *dif*

Two models have been put forward describing how recombination at *dif* could lead to chromosome resolution. In the first model, rapid unconstrained recombination between copies of *dif* creates an equilibrium between monomeric and dimeric chromosomes, until an active partition system causes physical chromosome separation. In the second model, all replicated chromosomes, whether monomeric or dimeric, undergo a single Xer catalysed strand exchange at *dif* to give a Holliday junction structure that persists until it is resolved in the correct manner to leave chromosomes in the monomeric form, possibly directed again by an active partition mechanism. These two models are described in Fig. 1.10

Several pieces of information support the hypothesis that *dif* is a chromosomal monomerisation locus. Phenotypic observation of *xerC* and *dif* cultures shows that only a small fraction of the cells in any sample are filamentous. These would correspond to the fraction of cells in which inter-chromosomal recombination has produced a chromosome dimer. Indeed, Kuempel *et al.*, (1991) present data calculating the frequency of recombination between chromosome copies that is consistent with their observed frequencies of filamentation in *dif* mutants. The induction of the SOS system is known to cause inhibition of cell division and filamentation, requiring the function of the *sfiB*

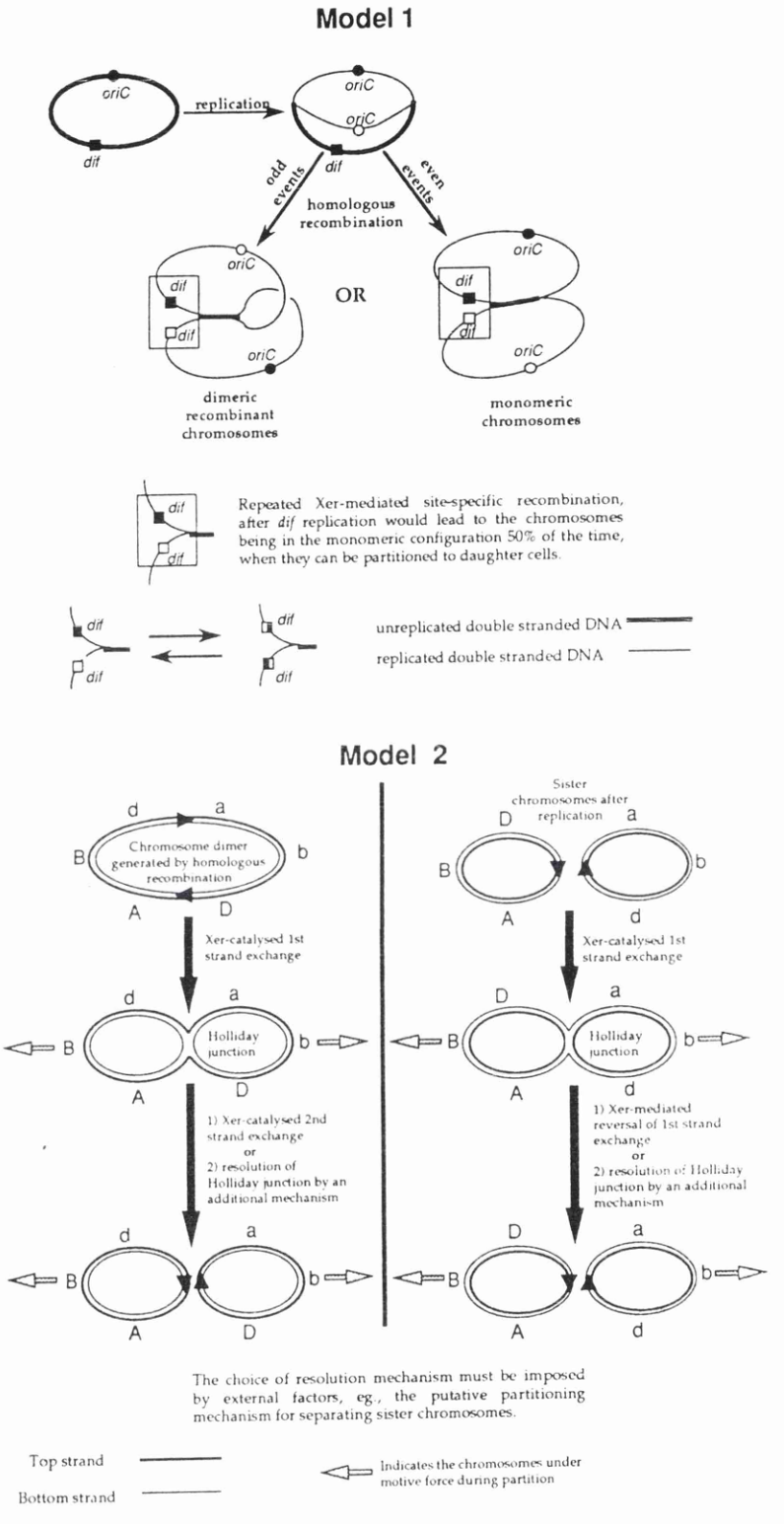


Figure 1.10. The role of *dif* recombination in chromosome monomerisation. Two possible models for the function of *dif* in chromosome monomerisation are presented. Each model is described in the main text. This diagram is adapted from Sherratt *et al.*, (1993).

(*ftsZ*) gene for this inhibition. Although this system is induced in *xerC* and *dif* mutants, a primary source of filamentation exists, as *dif sfiB* mutants produce filaments and some anucleate cells and have an aberrant distribution of nucleoids (Kuempel *et al.*, 1991). As would be predicted, the filamentation of *dif* and *xerC* mutants is suppressed by mutations in genes required for homologous recombination pathways. *xerC recA* mutants appear very similar to *recA* mutants, with a more normal distribution of nucleoid material than in *xerC* or *dif sfiB* mutants (Blakely *et al.*, 1991; Kuempel, 1991). Attempts to demonstrate the presence of dimeric chromosomes in *xerC* or *dif* mutants using pulse field gel electrophoresis have thus far been unsuccessful (G. Blakely, personal communication).

Chapter 2
Materials and Methods

2.1. Bacterial strains, plasmids and oligonucleotides.

Bacteria used were *Escherichia coli* K12 strains. Most were derived from AB1157 (Bachmann, 1972), which is *thr1 leuB6 hisG4 thi1 ara14 Δ(gpt-proA)62 argE3 galK2 supE44 xyl5 mtl1 tsx33 lacY1 rpsL31*. Details are shown in Table 2.1. In this table, strain construction described as "plasmid-X into strain-Y" refers to the introduction of mutations into the bacterial chromosome of strain-Y by transformation with linear DNA of plasmid-X (Fig. 3.1), with the exception of strains marked "†", into which mutations were introduced using a temperature sensitive plasmid replicon (Hamilton *et al.*, 1989, and Chapter 5). Constructions described as "strain-X into strain-Y" were performed by bacteriophage P1 transduction. Plasmids and oligonucleotides used are detailed in Tables 2.2 and 2.3 respectively. Oligonucleotides were synthesised on an Applied Biosystems 391 PCR Mate DNA Synthesiser.

2.2. Sources of materials

Chemicals	**Source**
General chemicals and solvents	BDH, Sigma
Media	Difco, Oxoid
Antibiotics, Xgal, XP, IPTG	Sigma
Agarose 'electrophoresis grade'	BRL (Ultrapure)
Restriction enzymes	BRL, NEB, Boehringer Mannheim
Modifying enzymes	BRL
Radiochemicals	NEN, ICN
Deoxynucleoside triphosphates	Promega
Oligonucleotide synthesis reagents	Cruachem Ltd., Applied Biosystems
Phenol	Fisons

Table 2.1 - Strains

Strain	Genotype	Reference/Construction
AB1157	(See Text)	Bachmann 1972
DS941	AB1157 *recF143, lacZΔM15, lacI*^{q}	Summers and Sherratt, 1988
DS902	AB1157 *recA13*	Bednarz *et al.*, 1990
JC7623	AB1157 *recB21C22 sbcB15C201*	Lloyd and Buckman 1985
V220	*recD1011 argA21 recF143 hisG4 met*	Amundsen *et al.*, 1986
DS956	DS941 *xerA9* (*argR*::Tp^{R})	Flinn *et al.*, 1989
DS957	DS941 *xerB1* (*pepA*::Tn5)	Stirling *et al.*, 1989
DS984	DS941 *xerC*::*Y17* (::mini Mu, Cm^{R})	Colloms *et al.*, 1990
DS981	DS941 *xerC2*(::Km^{R})	Colloms *et al.*, 1990
STL116	AB1157 *xerD2*(::mini Tn10-9, Km^{R})	Blakely *et al.*, 1993
DS9008	DS941 *xerD2*(::mini Tn10-9, Km^{R})	Blakely *et al.*, 1993
DL842	*mbf (lrp) 18*::mini Tn10	Braaten *et al.*, 1992
DL844	*mbf (lrp) 20*::mini Tn10	Braaten *et al.*, 1992
TH210	*tus*::Km^{R}	Hill *et al.*, 1989
SC5	JC7623 *xerC*::*Y17*	Colloms *et al.*, 1990
NL20	V220 *difΔ6*	pNΔ6 into V220
NL13	JC7623 *xerC*::*Y17 difΔ6*	pNΔ6 into SC5
NLΔINj	JC7623 *difΔ0IN*	pNΔ0IN into JC7623
NLΔOUTj	JC7623 *difΔ0OUT*	pNΔ0OUT into JC7623
NL40	DS941 *difΔ6*	NL20 into DS941
NL50	DS941 *xerC*::*Y17 difΔ6*	DS984 into NL40
NLΔIN	DS941 *difΔ0IN*	NLΔINj into DS941
NLΔOUT	DS941 *difΔ0OUT*	NLΔOUTj into DS941
NL60	DS941 *mbf (lrp) 18*	DL842 into DS941
NL70	DS941 *mbf (lrp) 20*	DL844 into DS941
NL80	AB1157 *difΔ6*	NL40 into AB1157
NL90	AB1157 *xerC*::*Y17*	DS984 into AB1157
NL124	JC7623 *xerC*::*Y17 difΔ6*::*res*Gm^{R}	pN24 into NL13
NL125	JC7623 *xerC*::*Y17 difΔ6*::*cer*Gm^{R}	pN25 into NL13
NL126	JC7623 *xerC*::*Y17 difΔ6*::33bp*dif*Gm^{R}	pN26 into NL13
NL127	JC7623 *xerC*::*Y17 difΔ6*::532bp*dif*Gm^{R}	pN27 into NL13
NL165	JC7623 *xerC*::*Y17 difΔ6*::*cer*Gm^{R}	pN65 into NL13
NL167	JC7623 *xerC*::*Y17 difΔ6*::532bp*dif*Gm^{R}	pN67 into NL13
NL166	JC7623 *xerC*::*Y17 difΔ6*::33bp*dif*Gm^{R}	pN66 into NL13
NL176	JC7623 *xerC*::*Y17 difΔ6*::33bp*dif*Gm^{R}	pN76 into NL13
NL169	JC7623 *xerC*::*Y17 difΔ6*::*cer6*Gm^{R}	pN69 into NL13
NL244	DS941 *difΔ6*::*res*Gm^{R}	NL124 into NL40
NL245	DS941 *difΔ6*::*cer*Gm^{R}	NL125 into NL40
NL246	DS941 *difΔ6*::minimal*dif*Gm^{R}	NL126 into NL40
NL247	DS941 *difΔ6*::532bp*dif*Gm^{R}	NL127 into NL40
NL285	DS941 *difΔ6*::*cer*Gm^{R}	NL165 into NL40
NL287	DS941 *difΔ6*::532bp*dif*Gm^{R}	NL167 into NL40
NL286	DS941 *difΔ6*::minimal*dif*Gm^{R}	NL166 into NL40
NL296	DS941 *difΔ6*::minimal*dif*Gm^{R}	NL176 into NL40
NL259	DS941 *pepA difΔ6*::*cer6*Gm^{R}	NL169 into DS957
NL279	DS941 *argR difΔ6*::*cer6*Gm^{R}	NL169 into DS956
NL289	DS941 *difΔ6*::*cer6*Gm^{R}	NL169 into NL40

Table 2.1 (..cont.)

NL290	DS941 *xerC*::*Y17 difΔ6*::532bp*dif*Gm^R	DS984 into NL287
NL132	NL13 *lacZ*::Gm^R	pN32 into NL13
NL137	NL13 *lacZ*::532bp*dif*Gm^R	pN37 into NL13
NL142	NL13 *Δ(pstCAB phoU)*::Gm^R	pN42 into NL13
NL147	NL13 *Δ(pstCAB phoU)*::532bp*dif*Gm^R	pN47 into NL13
NL232	NL50 *lacZ*::Gm^R	NL132 into NL50
NL233	DS941 *lacZ*::Gm^R	NL132 into DS941
NL237	NL50 *lacZ*::532bp*dif*Gm^R	NL137 into NL50
NL238	DS941 *lacZ*::532bp*dif*Gm^R	NL137 into DS941
NL250	NL40 *lacZ*::532bp*dif*Gm^R	NL137 into NL40
NL342	NL50 *Δ(pstCAB phoU)*::Gm^R	NL142 into NL50
NL343	DS941 *Δ(pstCAB phoU)*::Gm^R	NL142 into DS941
NL345	NL40 *Δ(pstCAB phoU)*::Gm^R	NL142 into NL40
NL347	NL50 *Δ(pstCAB phoU)*::532bp*dif*Gm^R	NL147 into NL50
NL348	DS941 *Δ(pstCAB phoU)*::532bp*dif*Gm^R	NL147 into DS941
NL350	NL40 *Δ(pstCAB phoU)*::532bp*dif*Gm^R	NL147 into NL40
NL208	NL40 *difΔ6*::*loxP*Gm^R	pN58 into NL40†
NL202	NL40 *lacZ*::*loxP*Gm^R	pN83 into NL40†
NL203	NL40 *Δ(pstCAB phoU)*::*loxP*Gm^R	pN84 into NL40†
NL98	JC7623 *trg*::*TerB*	pN94 into JC7623
NL100	AB1157 *trg*::*TerB*	NL98 into AB1157
NL102	DS941 *trg*::*TerB*	NL98 into DS941
NL106	AB1157 *tus*::Km^R	TH210 into AB1157

Table 2.2 - Plasmids

Plasmid	Description	Reference / Source
pUC8	Cloning vector	Vieira and Messing, 1982
pUC18 and pUC19	Cloning vectors	Yanisch-Perron *et al.* 1985
pMTL23	Cloning vector	Chambers *et al.* 1988
pACYC184	Cloning vector	Chang and Cohen , 1978
pCB104	λdv based vector	Boyd *et al.*, 1989
pBAD	P_{tac} expression vector	C. Boyd
pMAK705	Temperature sensitive pSC101 replicon vector	Hamilton *et al.* 1989
pLIM701	pMAK705 + *dif* for plasmid integration assay	Kuemple *et al.* 1991
pFC9	Temperature sensitive dif^+ plasmid	Cornet *et al.*, 1994
pGB310	pCB104 +*dif* core site fragment	G. Blakely
pBS12	Source of chromosomal *dif* region DNA	Bejar and Bouche, 1983
pSN518	Source of chromosomal *pstCAB phoU* DNA (pBR322 based)	Amemura *et al.* 1982
p357	Source of chromosomal *lacZ* DNA (pUC based)	M. Burke and D.J. Sherratt
pPM1000	Source of chromosomal DNA near *trg* gene	Moir *et al*, 1992
pUC71K	Source of 1.4kb Km^r fragment	Vieira and Messing, 1982
pGM160	Sourcr of 1.5kb Gm^r fragment	Muth *et al.* 1989
pPAK316	Transposition defective Tn3 in pACYC184	Kitts *et al.* 1983
pRH200	pUC based Cre expression plasmid	Mack *et al.*, 1992
pSD105	pUC based XerC expression plasmid	Colloms *et al.* 1990
pSD104	pUC based XerC expression plasmid	Colloms *et al.* 1990
pLA121	pSD104 based XerCY275F expression plasmid	Blakely *et al.* 1993
pLA130	pSD104 based XerCR243Q expression plasmid	L. Arciszewska

Table 2.2 (..cont.)

pRM130	pUC19 based XerD expression plasmid	Blakely *et al.* 1993
pLA137	pRM130 based XerDY279F expression plasmid	L. Arciszewska
pLA139	pRM130 based XerDE184K expression plasmid	L. Arciszewska
pLA127	pRM130 based XerDR247Q expression plasmid	Blakely *et al.* 1993
pMAY5	pUC based XerC, XerD expression plasmid	G. May
pEA305	λ*c*I repressor expression vector	Amann *et al*, 1983
pMIN33	33bp *dif* minimal oligonucleotides in pUC18	Blakely *et al.* 1991
pMA1441	282 bp Tn3 *res* site in pUC18	M. Boocock
pKS492	Full *cer* site in pUC18	Stirling *et al.*, 1988b
pTypeII	Full *cer6* site in pUC9	Summers, 1989
pLIM350	532bp *dif* fragment in pBR322	Kuemple *et al.* 1991
pMA21	Reporter plasmid. Two Tn3 *res* sites in direct repeat, Based on pBR322	Bednarz *et al.* 1990
pKS455	Reporter plasmid. Two *cer* sites in direct repeat, based on pUC9	Stirling *et al.* 1988b
pCS202	Reporter plasmid. Two *cer* sites in direct repeat, λdv based	Colloms *et al.*, 1990
pSD124	Reporter plasmid. Two *dif* sites in direct repeat, pUC18 based	Blakely *et al.*, 1991
pN1	pUC18 + 5.5 kb *Bam*HI *dif* fragment from pBS12	Chapter 4
pN30	pUC18 + 247bp *Sty*I-*Sau*3A *dif* fragment from pN1	Chapter 4
pN532	pUC18 + 532bp *Cla*I-*Sau*3A *dif* fragment from pN1	Chapter 4
pN20	pMTL23 + *Nde*I-*Hin*dIII *cer6* site fragment from pTypeII	Chapter 5
pN40	pMTL23 cut with *Hin*dIII + *loxP* site oligonucleotides O1 and O2	Chapter 5
pNΔ0 IN	pN1 cut with *Sty*I + 1.4kb Km^r fragment, transcribed towards *dif* site	Chapter 3
pNΔ0 OUT	pN1 cut with *Sty*I + 1.4kb Km^r fragment, transcribed away from *dif* site	Chapter 3
pNΔ15	pN1 cut with *Sty*I, exonuclease III treated (15 secs) + 1.4kb Km^r fragment	Chapter 3

Table 2.2 (..cont.)

pNΔ30	pN1 cut with *Sty*I, exonuclease III treated (30 sces) + 1.4kb Km^r fragment	Chapter 3
pNΔ1	pN1 cut with *Sty*I, exonuclease III treated (1 min) + 1.4kb Km^r fragment	Chapter 3
pNΔ4	pN1 cut with *Sty*I, exonuclease III treated (4 mins) + 1.4kb Km^r fragment	Chapter 3
pNΔ6	pN1 cut with *Sty*I, exonuclease III treated (6 mins) + 1.4kb Km^r fragment	Chapter 3
pN7	pLIM350 + 1.5kb *Bam*HI-*Pvu*II Gm^r fragment from pGM160	Chapter 4
pN10	pMTL23 + 1.4kb *Hin*dIII Gm^r fragment from pGM160	Chapter 4
pN12	pMTL23 + 1.4kb *Hin*dIII Gm^r fragment from pGM160 (opposite orientation)	Chapter 4
pN14	pN10 + *Kpn*I-*Xba*I *res* site fragment from pMA1441	Chapter 5
pN15	pN10 + *Bam*HI-*Pst*I *cer* site fragment from pKS492	Chapter 5
pN16	pN10 + *Bam*HI-*Pst*I minimal *dif* site fragment from pMIN33	Chapter 4
pN24	pNΔ6 cut with *Xho*I and *Nru*I + *Nae*I-*Xho*I *res* Gm^r fragment from pN14	Chapter 5
pN25	pNΔ6 cut with *Xho*I and *Nru*I + *Nae*I-*Xho*I *cer* Gm^r fragment from pN15	Chapter 5
pN26	pNΔ6 cut with *Xho*I and *Nru*I + *Nae*I-*Xho*I *dif* Gm^r fragment from pN16	Chapter 4
pN27	pNΔ6 cut with *Cla*I and *Nru*I + *Pvu*II-*Cla*I *dif* Gm^r fragment from pN7	Chapter 4
pN55	pN15 cut with *Eco*RI and *Pst*I + Gm^r fragment from pN12 (opposite orientation)	Chapter 5
pN57	pN12 + *Bam*HI-*Pst*I 532bp *dif* fragment from pN532	Chapter 4
pN65	pNΔ6 cut with *Xho*I and *Nru*I + *Nae*I-*Xho*I *cer* Gm^r fragment from pN55	Chapter 5
pN67	pNΔ6 cut with *Xho*I and *Nru*I + *Nae*I-*Xho*I *dif* Gm^r fragment from pN57	Chapter 4
pN36	pN12 + *Bam*HI-*Pst*I minimal *dif* fragment from pMIN33	Chapter 4
pN66	pNΔ6 cut with *Xho*I and *Nru*I + *Nae*I-*Xho*I *dif* Gm^r fragment from pN36	Chapter 4
pN76	pNΔ6 cut with *Cla*I and *Nru*I + *Nae*I-*Cla*I *dif* Gm^r fragment from pN36	Chapter 4
pN38	pN40 cut with *Bgl*II and *Eco*RI + *Bam*HI-*Eco*RI Gm^r fragment from pN10	Chapter 5
pN39	pN20 cut with *Xho*I and *Eco*RI + *Sal*I-*Eco*RI Gm^r fragment from pN10	Chapter 5

Table 2.2 (..cont.)

pN68	pNΔ6 cut with *Nru*I + *Nae*I-*Nru*I *loxP* Gm^r fragment from pN38	Chapter 5
pN69	pNΔ6 cut with *Nru*I + *Nae*I-*Nru*I *cer6* Gm^r fragment from pN39	Chapter 5
pN32	p357 cut with *Sst*I and *Bcl*I + *Sst*I-*Bam*HI Gm^r fragment from pN12	Chapter 7
pN37	p357 cut with *Sst*I and *Bcl*I + *Sst*I-*Bam*HI *dif* Gm^r fragment from pN57	Chapter 7
pN42	pSN518 cut with *Ssp*I + *Stu*I-*Nae*I Gm^r fragment from pN12	Chapter 7
pN47	pSN518 cut with *Ssp*I + *Stu*I-*Nae*I *dif* Gm^r fragment from pN57	Chapter 7
pN81	p357 cut with *Eco*RV + *Nae*I-*Nru*I *loxP* Gm^r fragment from pN38	Chapter 7
pN82	pSN518 cut with *Ssp*I + *Nae*I-*Nru*I *loxP* Gm^r fragment from pN38	Chapter 7
pN58	pMAK705 cut with *Kpn*I + *Kpn*I terminus DNA *loxP* Gm^r fragment from pN68	Chapter 5
pN83	pMAK705 cut with *Hin*cII + *Pvu*II *lacZ* DNA *loxP* Gm^r fragment from pN81	Chapter 7
pN84	pMAK705 cut with *Hin*cII + *Pvu*II *pst* DNA *loxP* Gm^r fragment from pN82	Chapter 7
pN78	pMAK705 + *Pst*I-*Xba*I *loxP* site from pN40	Chapter 5
pN79	pMAK705 + *Bam*HI-*Sst*I *cer6* site fragment from pN20	Chapter 5
pN85	pUC8 cut with *Pst*I + 8.0 kb *Pst*I fragment from pPM1000	Chapter 7
pN90	pUC18 cut with *Asp*718 and *Sst*I + *Ter* site oligonucleotides O3 and O4	Chapter 7
pN91	pUC19 cut with *Asp*718 and *Sst*I + *Ter* site oligonucleotides O3 and O4	Chapter 7
pN93	pN10 cut with *Eco*RI and *Kpn*I + *Eco*RI-*Kpn*I *Ter* fragment of pN90	Chapter 7
pN94	pN85 cut with *Eco*RV and *Kpn*I + *Nae*I-*Kpn*I *Ter* Gm^R fragment of pN93	Chapter 7

Table 2.3 -Oligonucleotides

Top and bottom strand *loxP* site oligo's for production of pN40

O1
5′ AGCTCGAGATAACTTCGTATAATGTATGCTATACGAAGTTAT 3′

O2
5′ AGCTATAACTTCGTATAGCATACATTATACGAAGTTATCTCG 3′

Top and bottom strand *Ter* site oligo's for production of pN90 and pN91

O3
5′ GTACCATAAAATAAGTATGTTGTAACTAAAGTACTGAGCT 3′

O4
5′ CAGTACTTTAGTTACAACATACTTATTTTATG 3′

Top strand *dif* oligo' for Southern hybridisation

O5
5' CTAGATTGGTGCGCATAATGTATATTATGTTAAATCAG 3′

Oligonucleotides O1 and O2 were kindly provided by Marshall Stark.
Oligonucleotide O5 was kindly provided by Garry Blakely.

2.3. Bacterial growth conditions. Unless otherwise stated, bacteria were grown at 37° either in LB broth (Miller, 1972) with vigorous shaking, or on LB agar plates (LB broth + 15g/l agar). However, where indicated, YT broth (Sambrook *et al.*, 1989), minimal media or BBL media were used. Minimal media contained 1x Davis and Mingioli salts, with either 0.4% glucose and additional amino acids (normally those required by AB1157 and its derivatives) or 5% casamino acids solution (Difco).

4x Davis and Mingioli salts contains 28g $K_2HPO_4.3H_2O$, 8g KH_2PO_4, 4g $(NH_4)_2SO_4$, 1g tri-sodium citrate.$2H_2O$ and 0.4g $MgSO_4.7H_2O$ made up to 1 litre with de-ionised water.

BBL media contain 10g trypticase peptone and 5g NaCl made up to 1 litre with de-ionised water, and adjusted to pH 7.2 with NaOH. 2.5g of $MgSO_4.6H_2O$ is added before solidification with agar or agarose.

Antibiotics and other supplements were added to media as appropriate. The antibiotic concentrations used were as follows.

Antibiotic	**Selective concentration**
Ampicillin	50μg/ml
Kanamycin	25μg/ml
Gentamicin	5μg/ml
Chloramphenicol	25μg/ml
Tetracycline	10μg/ml
Streptomycin	100μg/ml
Spectinomycin	25μg/ml

Long term storage of bacterial strains was at -70°, as an equal mixture of stationary phase culture and a 40% glycerol, 1% peptone solution.

2.4. Bacterial transformation. The preparation of competent cells by treatment with $CaCl_2$, and transformation with plasmid DNA are described in Sambrook *et al.*, (1989).

2.5. Preparation of DNA.

i. Single colony lysis method. This method allows fast, crude analysis of the plasmid content of a population of cells. Although normally performed using a large single colony, or a small patch of cells derived from one, this method was also used with cells harvested from a liquid culture. Cells were resuspended in 100-200µl of single colony buffer (10% ficoll, 5% SDS, 0.25% Orange G, 0.05% Bromophenol Blue in 1xTAE buffer (Sambrook *et al.*, 1989)), allowed to lyse at room temperature for 30 minutes, then spun for 30 minutes in a microfuge at 14,000 rpm. Plasmid DNA in the supernatant could then be visualised by agarose gel electrophoresis and staining with ethidium bromide.

ii. Small scale plasmid preparations. Small quantities of plasmid DNA suitable for further *in vitro* manipulations were prepared by the method of Sambrook *et al.*, (1989). A phenol/chloroform extraction step was usually included before isopropanol precipitation.

iii. Large scale plasmid preparations. Larger quantities of plasmid DNA for *in vitro* manipulation and long term storage were prepared using 'Qiagen-tip 100' columns following the manufacturers instructions.

iv. Preparation of bacterial genomic DNA. Chromosomal DNA was prepared using the method of Neumann *et al.*, (1992). Centrifugation before

isopropanol precipitation was performed at 12,000 rpm, not 4,000 rpm as recommended.

2.6. *in vitro* DNA manipulations. These were performed largely as described in Sambrook *et al.*, (1989)

i. Restriction digests. DNA was cut by incubation with restriction enzymes, according to the manufacturers instructions. Restriction enzymes were used at a concentration of approximately 0.5 units/µl in reaction buffers supplied with the enzymes (generally React buffers from BRL).

ii. Filling-in and ligation of DNA ends. Where necessary, the ends of DNA cut with restriction enzymes were filled in using the Klenow fragment of *E.coli* DNA polymerase I and dNTPs. DNA ends were ligated using T4 DNA ligase (≈.05 units/µl) in 1x ligase buffer (BRL). Incubation was performed overnight (≈15 hrs) at 16°.

iii. Preparation of radio-labelled probes. Radioactive labelling of plasmid DNA restriction fragments was performed using a Random Primed DNA Labelling Kit (Boehringer Mannheim). Radioactive labelling of synthetic oligonucleotides was performed using [γ-^{32}P]ATP and 'phage T4 polynucleotide kinase, as described by Sambrook *et al.*, (1989). Before use, labelled probes were purified using Nuc-Trap purification columns (Biorad), following the manufacturers instructions.

2.7. Agarose gel electrophoresis. DNA samples were analysed on horizontal agarose gels using the methods of Sambrook *et al.*, (1989). Gels were routinely 1% BRL agarose in 1xTAE buffer. IBI 'midi', and larger custom made gel kits were used. Samples were loaded in Ficoll containing loading buffers, and gels

run in 1xTAE buffer (Sambrook *et al.*, 1989) using Kikusui PAB power packs. Gels were stained with ethidium bromide (0.5μg/ml in running buffer) for 30 mins and destained in tap water for 30 mins, and then visualised using either a 245 nm or 365 nm UV transilluminator. Gels were photographed using a Pentax 35mm SLR camera and Kodak Wratten UV filter No. 23A with Ilford HP5 film (400 ASA).

DNA restriction fragments were purified from agarose gels using SPIN-X filtration columns (Costar, Cambridge, MA, USA), following the manufacturers instructions. If DNA was to be recovered from gels, only a long-wave (365 nm) transilluminator was used.

2.8. Southern blotting and hybridisation with radio-labelled probes.

Unless otherwise stated, this was performed using Hybond-N membranes (Amersham), largely as described in manufacturers instructions, and in Sambrook *et al.*, (1989). Denatured and depurinated DNA was transferred from agarose gels onto Hybond-N by neutral pH capillary blotting and fixed onto the membrane by UV crosslinking, using a Stratalinker (Stratagene). DNA-DNA Hybridisation was performed at 65° when using large restriction fragments as probes. When using labelled oligonucleotides as probes, hybridisation and washing temperatures were calculated by the methods of Sambrook *et al.*, (1989) and Miyada and Wallace, (1987), membranes being initially washed twice in 6xSSC solution at approximately 35° for 15 mins, then washed once in 2xSSC at 65° for 2 mins. Membranes hybridised with large DNA fragments were washed as described in Amersham protocols before autoradiography. Autoradiography used Fuji RX X-ray film.

2.9. Microscopy. Microscopic analysis of nucleoid morphology by staining with DAPI followed the methods of Hiraga *et al.* (1989) and Eliasson *et al.*, (1992). Cell nucleoids were generally condensed with chloramphenicol (250

μg/ml) at 37° for 10-20 minutes before cells were washed, dried onto microscope slides, fixed with cold methanol (-20°), washed again in tap water and stuck onto the slide surface using poly-L-lysine. Nucleoids were then stained by addition of 1μg/ml DAPI (4',6-diamidino-2-phenylindole) in 30% glycerol/70% iso-tonic saline solution (0.84% NaCl). These preparations were examined using combined phase contrast and fluorescence microscopy. Preparations were found to exhibit good nucleoid staining for as long as two years if stored in the dark.

Microscopy used a Leitz Orthoplan microscope. A Ploemopak fluorescence vertical illuminator or Nomarski optics were also used for some work. Photomicrographs were taken using Ilford HP5 film, or Kodacolour Gold film (both 400 ASA).

2.10. Generalised transduction. Chromosomal mutations including selectable markers were introduced into new strains by bacteriophage P1 transduction, using a method adapted from Miller, (1972).

i. Preparation of lysates. The cells from 200 μl of a stationary phase culture of the donor strain were resuspended in 100 μl of LB broth. To this, 100 μl of $MgCl_2$ (100mM), 100 μl $CaCl_2$ (50mM) and a lysate of bacteriophage $P1_{kc}$ were added. Generally, three independent infections were performed using different volumes of a previous lysate (0.5 μl - 150 μl). Cells were incubated at 37° for 25 mins, before mixing with BBL top-agarose (40°) and plating onto undried BBL agar plates. After overnight incubation, 2.5 mls of 'phage buffer was poured onto plates which were then left for 15 mins at room temperature. The 'phage buffer and top-agarose were mixed vigorously with 150 μl of chloroform for 30 secs, and left for 30 mins at room temperature, before vortexing again. Centrifugation at 12,000 rpm for 10 minutes allowed

the removal of the lysate for storage in air-tight containers with more chloroform (150µl) at 4°.

ii. Transduction. The cells from 200µl of an exponentially growing culture of the recipient strain were resuspended in 100µl of LB broth. To this, 100 µl of $MgCl_2$ (100mM), 100 µl $CaCl_2$ (50mM) and a donor strain lysate (0.5 µl - 50 µl) were added. Cells were incubated for 20 mins at 37° before the addition of 200µl of sodium citrate (1M, filter sterilised). 500µl of 2xYT broth was added and cells incubated at 37° for 90 mins to allow the expression of antibiotic resistance genes, before plating onto selective media.

2.11. Plasmid integration assays. This method was adapted from Kuempel *et al.*, (1991). Strains to be tested were transformed with pMAK705 or derivative plasmids, and grown into stationary phase in 2xYT broth with chloramphenicol at 30°. Cells were then diluted and plated onto pre-warmed chloramphenicol plates at 30° and 42°. Colonies were counted after 24 hours of growth. The number of colonies formed at 42° divided by the number at 30° was taken to be the fraction of transformed cells containing a plasmid copy integrated into the chromosome.

2.12. Cre recombination *in vitro*. The recombinatory activity of *loxP* sites in plasmids used in experiments was verified by *in vitro* recombination assays using purified Cre recombinase. Purified Cre recombinase was a kind gift of Andy Bates, at the University of Liverpool and Marshall Stark. Plasmids to be tested for *loxP* activity were incubated at 37° in React 2 buffer (BRL) for an hour with and without 0.5µl of diluted Cre preparation, before analysis of plasmid DNA by agarose gel electrophoresis. Cre mediated recombination *in vitro* is described by Sternberg *et al.*, (1983).

2.13 Computers. All software was run using a variety of Apple Macintosh computers, and all printing used an Apple Personal LaserWriter. All database analysis and sequence comparison used the GCG (Genetics Computer Group) package from the University of Wisconsin, available through the Glasgow University Unix computer. Statistical analysis, including the performance of one way analyses of variance was carried out using the Minitab statistics programme. Word processing and production of graphics used Microsoft Word, Claris MacDraw Pro and MacPlasmap.

Chapter 3

Deletion of the chromosomal *dif* site and comparative analysis of the *dif/xer* phenotype

Introduction - Deletion of the chromosomal *dif* site and comparative analysis of the *dif/xer* phenotype

After the work of Kuempel *et al.*, (1991) and Blakely *et al.*, (1991), many questions about the biological function of *dif* arose. If *dif* is a chromosomal resolution site, how does it achieve this, and could more evidence be produced to support this hypothesis? Are sequences required beyond the 33 bp fragment known to be a substrate for plasmid recombination? Is the location of *dif* in the replication terminus vital for its function? Many experiments approaching these questions require the construction of a chromosomal *dif* mutant as a pre-requisite. This would also allow direct phenotypic comparisons between strains carrying mutations of *dif*, *xerC* and the newly characterised *xerD* gene.

3.1 Construction of mutant bacterial strains

The construction of desired nucleotide sequence combinations and the introduction of these mutations into the bacterial chromosome is central to many of the experiments described in this thesis. In order to easily introduce specific sequences at specific locations in the chromosome, it was necessary to construct mutant alleles on plasmid vectors, then subsequently cross these mutations into the chromosome by homologous recombination. Several such methods of chromosomal mutagenesis rely on selecting for the integration of a mutation bearing plasmid, by inhibiting plasmid replication, and subsequently screening for deletion of the original wild-type sequence (Gutterson and Koshland, 1983; Flinn *et al.*, 1989; Hamilton *et al.*, 1989). However, the process of screening colonies for deletion of the wild-type allele, usually by looking for antibiotic sensitivity, can be lengthy, particularly when the mutagenesis results in reduced

growth and viability, as in the case of the deletion of the chromosomal *dif* site. Therefore in these studies, mutations were introduced into the chromosome by a mechanism that deletes all plasmid vector sequences, through transformation of strains deficient in exonuclease V with linear plasmid DNA and selection for a marker gene contained within the region of chromosomal homology (Jasin and Schimmel, 1984; Winans *et al.*, 1985; Russel *et al.*, 1989; Fig. 3.1). This technique has the advantage that the linearity of the incoming DNA ensures that both homologous exchanges required for allelic replacement occur in one step, and that only a small fragment of the mutagenic plasmid, flanked on both sides by chromosomal homology is inserted into the chromosome. The most frequently used recipient strain was JC7623 (*recB recC sbcB sbcC*, Kushner *et al.*, 1971; Lloyd and Buckman, 1985) although V220 (*recD*, Amundsen *et al.*, 1986) was also used. In *recB recC* mutants, further mutations in *sbcB* and *sbcC* are required to restore recombination proficiency. This technique has the advantage not only that incoming linear DNA appears not to be efficiently degraded, allowing transformation, but that JC7623 is an extremely poor host strain for the cloning vectors used (Cohen and Clark, 1986). This greatly reduces the probability of antibiotic resistance being carried on an uncut or re-ligated plasmid, facilitating selection for target mutants. That mutations had indeed replaced the natural chromosomal locus could be verified by demonstrating ampicillin sensitivity, by finding no detectable plasmid DNA content by the single colony lysis method, and by Southern hybridisation. However, JC7623 and strains derived from it are highly filamentous and shows greatly reduced viability (Chaudhury and Smith, 1984). Therefore, in order to have all constructs in the same, healthy, non-filamentous strain background, mutations were transferred into a DS941 background (*recF lacZΔM15 lacI^q*) by bacteriophage P1 transduction. JC7623 and its derivatives can be distinguished from the majority of strains used in these experiments (DS941 and derivatives) as they are morphologically filamentous, and are *lacZ*$^+$. Therefore, when grown on LB plates containing X-gal and IPTG

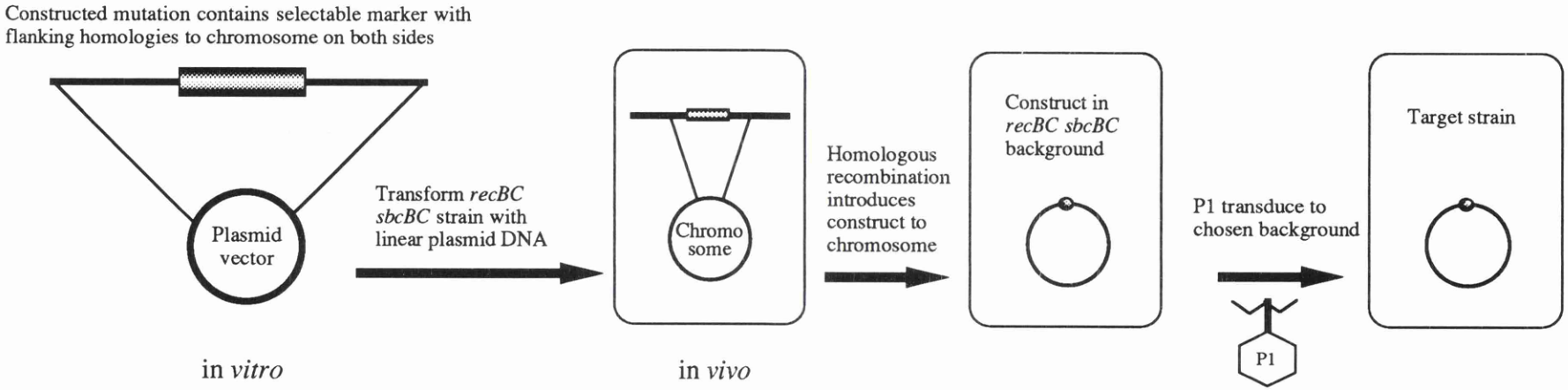


Figure 3.1. The strategy used for the introduction and deletion of recombination sites into and from the *E. coli* chromosome. Desired constructs containing an antibiotic resistance gene and flanked on both sides by homology to the chromosome were produced in *vitro*, and this DNA, linearised, was used to transform *recBC sbcBC* cells (represented by a rounded-cornered rectangle). Linear transformation was first described by Winans *et al.*, (1984) and Jasin and Schimmel, (1985). P1 transduction was subsequently used to transfer mutations to a desired genetic background.

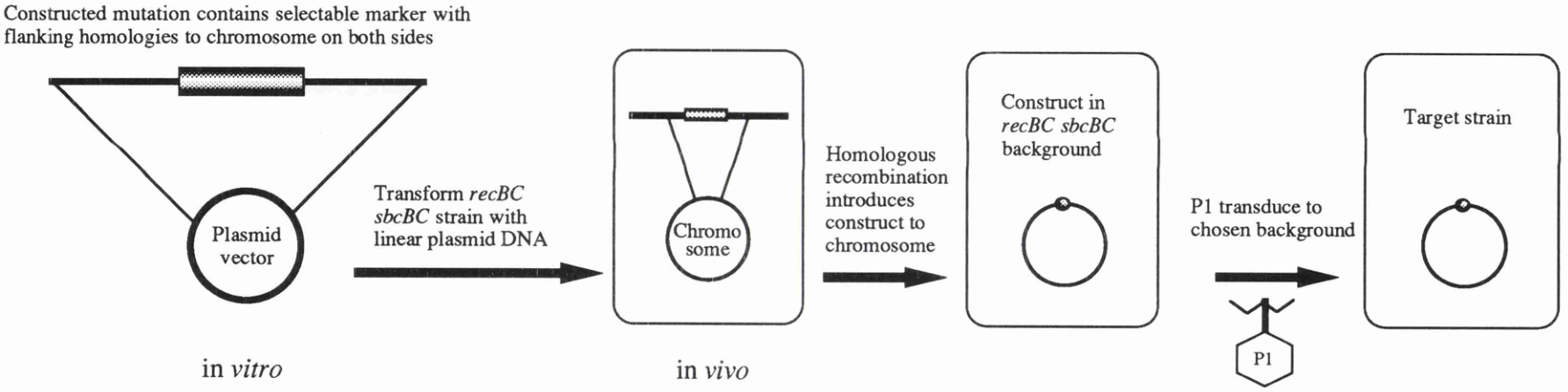


Figure 3.1. The strategy used for the introduction and deletion of recombination sites into and from the *E. coli* chromosome. Desired constructs containing an antibiotic resistance gene and flanked on both sides by homology to the chromosome were produced in *vitro*, and this DNA, linearised, was used to transform *recBC sbcBC* cells (represented by a rounded-cornered rectangle). Linear transformation was first described by Winans *et al.*, (1984) and Jasin and Schimmel, (1985). P1 transduction was subsequently used to transfer mutations to a desired genetic background.

(both at 20μg/ml), JC7623 and derivatives form blue colonies, whereas DS941 and derivatives form white colonies (Materials and Methods; Miller, 1972). JC7623 and its derivatives can also be easily differentiated from filamentous DS941 derivatives (*xer* and *dif* mutants) microscopically, as they display uniform filamentation with far less cell length variability. A brief cartoon of the strategy used in the construction of mutants is shown in Fig. 3.1.

3.2 Deletion of the chromosomal *dif* site

A *dif* mutant strain was produced by deletion of *dif* from an appropriate *E. coli* genomic DNA fragment carried on a plasmid, and replacement of the natural chromosomal locus with this construct by permanent homologous recombination (Fig. 3.1). In order that deletants could be selected for, the plasmid constructs contained a kanamycin resistance gene in place of *dif*.

The chromosomal *dif* locus of *E. coli* is located in the replication terminus region, at minute 33.6 of the genetic linkage map, kilobase 1608 of the physical map (Kohara *et al.*, 1987; Bachmann, 1990; Kuempel *et al.*, 1991). Throughout this study, information regarding the position of restriction enzyme sites around the *dif* locus was taken from the physical map of Kohara *et al.*, (1987) and the nucleotide sequence of Black *et al.*, (1991). pBS12 (Béjar and Bouché, 1983) was used as a source of DNA containing the *dif* locus. As this plasmid is approximately 33 kb in size, a 5.5 kb *Bam*HI fragment with *dif* centrally located was sub-cloned into pUC18 to give pN1 (Fig. 3.2). In order that a range of different *dif*⁻ constructs would be available for future work, deletion was performed using a time-course of exonuclease III digestion. pN1 contains two *Sty*I sites, 367 bp apart, one being 7 bp away from the XerC binding arm of the *dif* core recombination site, as shown in Fig. 3.2 and fig. 3.4. Digestion of pN1 with *Sty*I therefore removes this 367 bp fragment leaving the core site intact, and

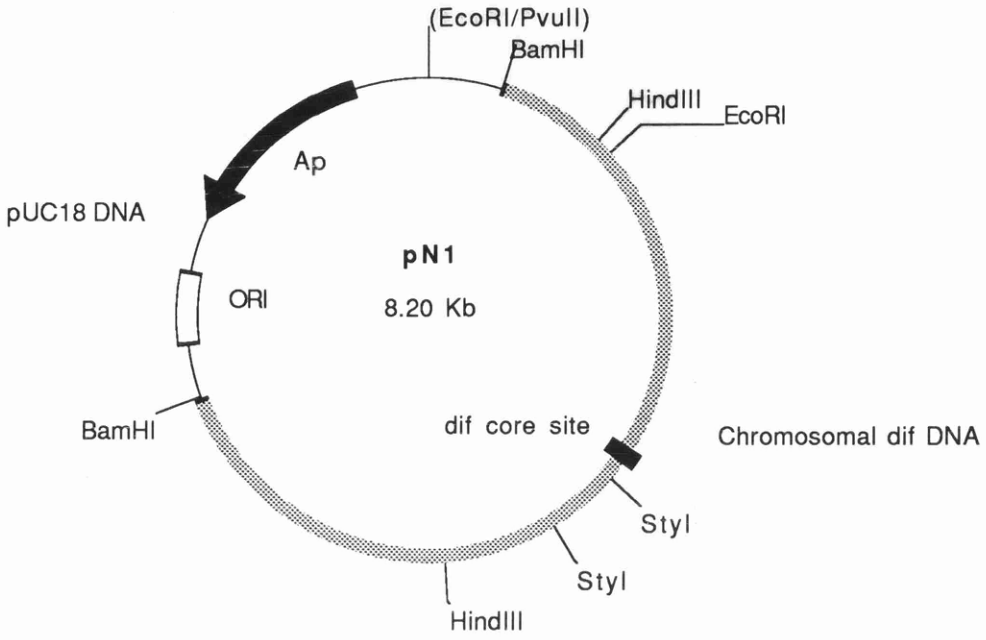


Figure 3.2. Plasmid pN1. A 5.5 kb *Bam*HI fragment from pBS12 (Bejar and Bouché, 1983) was cloned into the *Bam*HI site of pUC18, to give pN1. This is a 5.5 kb chromosomal fragment from the replication terminus region of *E.coli*, with the *dif* core recombination site centrally located. This plasmid was used in future manipulations of *dif* locus DNA and in the construction of mutant strains. Chromosomal DNA is represented by a thick shaded line and pUC18 DNA by a thin black line (including boxes denoting the *bla* gene and the origin of replication).

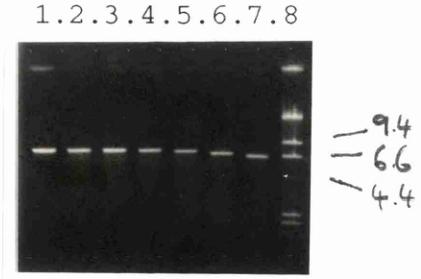


Figure 3.3. Digestion of plasmid pN1 DNA with exonuclease III. Plasmid DNA of pN1 was digested with *Sty*I and a time-course of exonuclease III digestion performed at 37°. Analysis by agarose gel electrophoresis gave the gel opposite. Samples were as follows,

1. *Sty*I digested only
2. 15 seconds of exonuclease III digestion
3. 30 secs
4. 1 min
5. 2 mins
6. 4 mins
7. 6 mins
8. Size markers (sizes are marked in kilobases)

This gel shows the reduction in size caused by exonuclease activity, upto a maximum deletion of 1500 bp at the 6 minute timepoint. These DNA samples were used to produce *dif* deleted plasmids as described in the main text.

allows entry for exonuclease III. A time course of bi-directional exonuclease III digestion was then performed on this DNA; samples were removed after incubation with the enzyme for 15 sec, 30 sec, 1 min, 4 min, and 6 min at 37° (Fig. 3.3). By ligation to a 1.5 kb Km^R *Bam*HI fragment from pUC71K (Pharmacia), plasmids were produced, pNΔ15, pNΔ30, pNΔ1, pNΔ4, and pNΔ6 respectively. Two pNΔ0 plasmids were made by ligation of the kanamycin resistance marker to *Sty*I cut pN1 DNA without digestion with exonuclease III, pNΔ0IN and pNΔ0OUT. These two plasmids retain a *dif* core site, and vary only in the transcriptional orientation of the kanamycin resistance gene relative to the surrounding DNA. Restriction enzyme analysis of all deleted plasmids showed that deletions extending from the initial sites of *Sty*I digestion were of approximately equal size in both directions, up to a total maximum size of approximately 1500 bp (pNΔ6, Fig. 3.5).

This work produced a range of plasmids containing the sequences flanking the natural chromosomal *dif* locus, with deletions of various sizes centred on the *Sty*I sites next to the *dif* core site, marked by a kanamycin resistance gene (see e.g.. Figs. 3.5, 3.4 and 4.4). The deletion of the *dif* core site was verified by Southern hybridisation of an end labelled oligonucleotide of that sequence to each plasmid (Fig. 3.6).

Deletion constructs were transferred to the bacterial chromosome from pNΔ0IN, pNΔ0OUT, pNΔ30 and pNΔ6, giving NLΔIN, NLΔOUT, NL41 and NL40 respectively, by transformation of strains deficient in exonuclease V with linearised plasmid DNA and selection for kanamycin resistance (as described above). The chromosomal *dif* region mutations in these strains were verified by Southern hybridisation of a radio-labelled *dif* core site oligonucleotide to chromosomal DNA digested with restriction enzymes (see Figs. 4.6, 5.7 and 7.3) Information regarding the strains created and each mutation used is contained in the following Table 3.1, and in Fig. 3.4

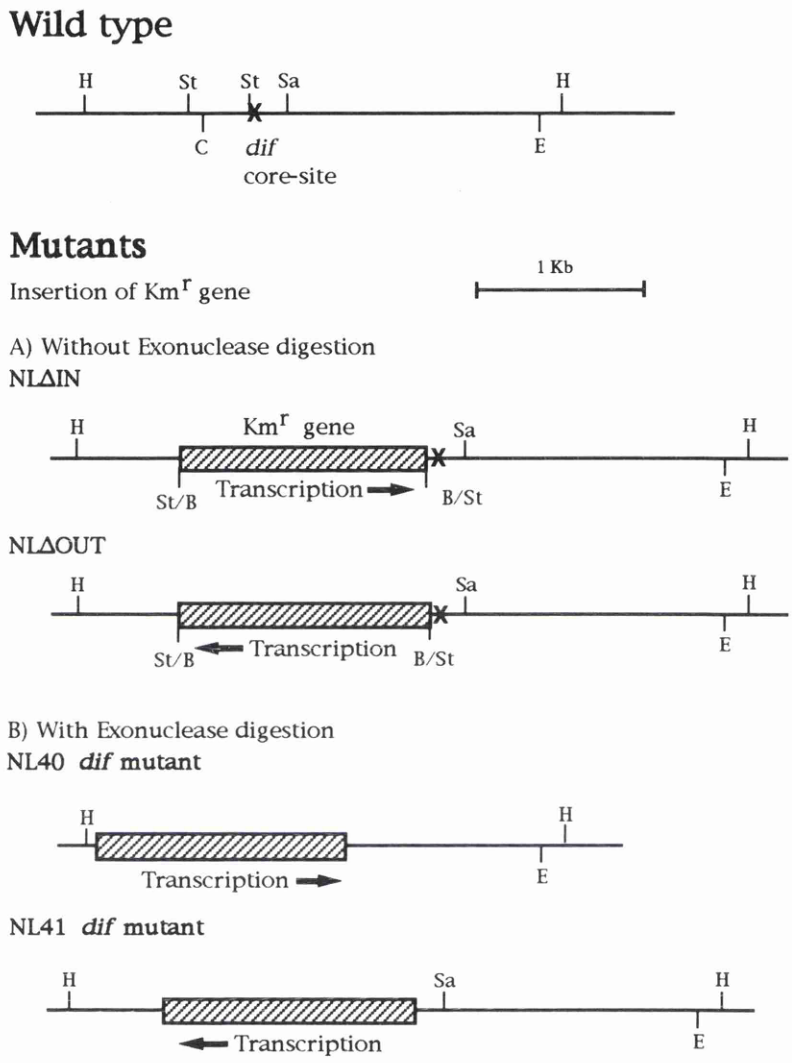


Fig. 3.4. Construction details of *dif* mutant strains. Diagrams represent the chromosomal *dif* region. The core site, represented by a cross, corresponds to the 28 bp XerC and XerD binding region shown to be a sufficient substrate for plasmid recombination. The hatched boxes represent the entire kanamycin resistance cassette including promoters. Restriction enzyme sites are labelled E - *Eco*RI, H - *Hin*dIII, C - *Cla*I, St - *Sty*I, Sa - *Sau*3A, B - *Bam*HI. The distances between restriction sites are approximately to scale, with the exception of the separation of the closest *Sty*I site from the XerC binding site of *dif*. This should be only 7 bp of DNA, but is shown as more for the sake of clarity.

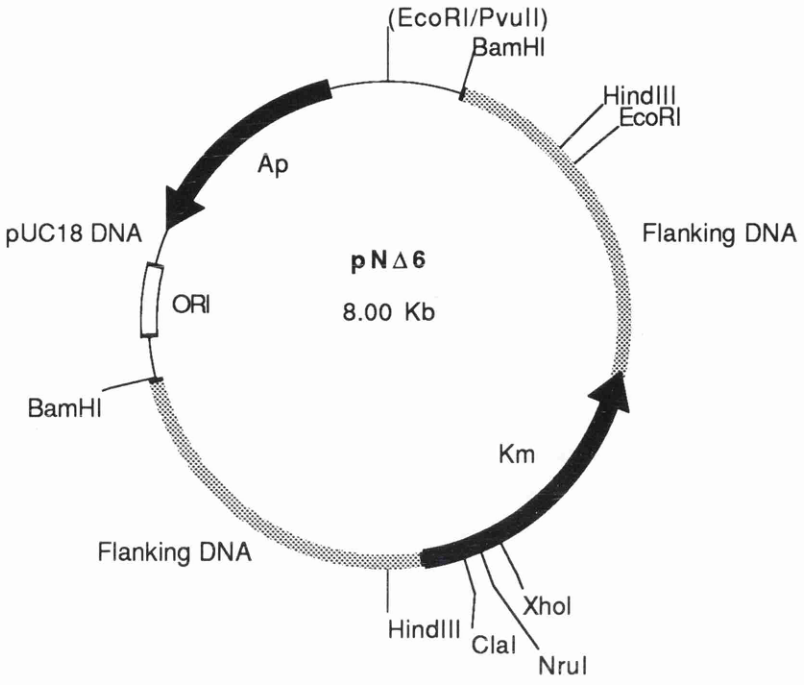


Fig. 3.5. Plasmid pNΔ6. Plasmid pN1 (Fig. 3.2) was cut with *Sty*I, treated with exonuclease III and the resulting bi-directional deletion sealed by ligation to a kanamycin resistance gene, as described in the text (Chapter 3). The resulting plasmid pNΔ6 harbours a deletion of the chromosomal *dif* locus (the mutation *difΔ6*) of approximately 1500 bp, with chromosomal DNA flanking the kanamycin gene on both sides. pNΔ6 was used in the construction of the *dif* deleted strain NL40 (Fig. 3.4), and in many further manipulations of the chromosomal *dif* locus, including the re-introduction of sequences into this deletion. Antibiotic resistance genes are represented by black arrowed boxes, DNA from the chromosomal *dif* region as a thick shaded line, and pUC18 DNA as a thin black line.

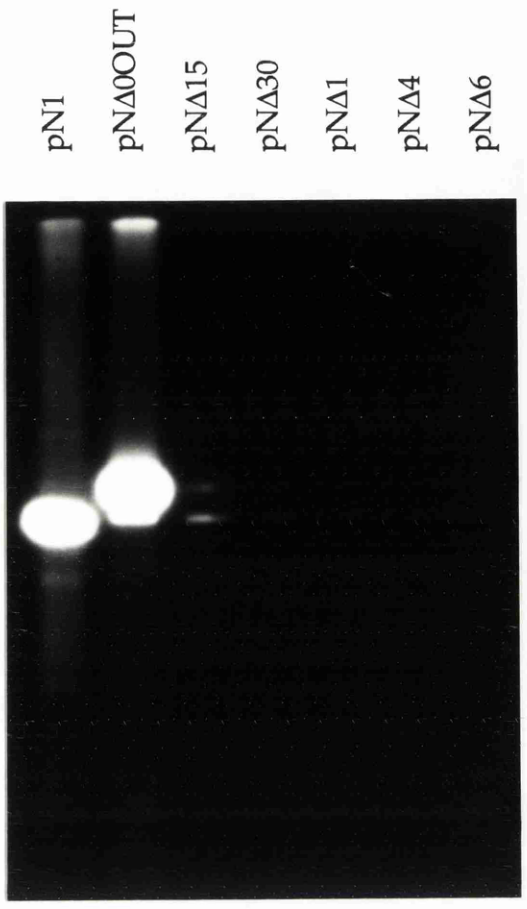


Figure 3.6. The *dif* core site sequence has been deleted by treatment with exonuclease III. A series of exonuclease III digestions were performed, deleting the *dif* site sequences from plasmid pN1 (Fig. 3.2), giving plasmids pNΔ15, pNΔ30, pNΔ1, pNΔ4 and pNΔ6 (see main text and Fig. 3.5). Southern hybridisation with a labelled *dif* core site oligonucleotide was used to demonstrate the deletion of these sequences from these plasmids. Equal quantities of each plasmid DNA was digested with *Bam*HI, before separation on an agarose gel and binding to a synthetic membrane by capillary blotting. Hybridisation with a radiolabelled *dif* core site probe, followed by autoradiography gave the hybridisation seen above. Only pN1 and pNΔ0OUT appear to retain full *dif* core sites, whilst pNΔ15 may retain some core site sequence.

Table 3.1

dif mutation	Original plasmid	Duration of exonuclease digestion	Total size of deletion	*dif* Core site	Derived strain
Δ0IN	pNΔ0IN	0	367 bp	+	NLΔIN
Δ0OUT	pNΔ0OUT	0	367 bp	+	NLΔOUT
Δ30	pNΔ30	30 sec.	≈430 bp	-	NL41
Δ6	pNΔ6	6 min.	≈1500 bp	-	NL40

3.3 The *dif/xer* phenotype

Kuempel *et al.*, (1991), show that *dif* mutants display a novel morphological phenotype, in which some cells in a culture are obviously filamentous, whereas most are morphologically normal. They note that half of the total cell mass of the culture is contained in approximately 10% of the cells, and that the SOS system is highly induced. By fluorescence microscopy, staining cell nucleoids with 4', 6-diamidino-2-phenylindole (DAPI), they also show that the morphology of the nucleoids in these filaments is aberrant. Some filamentous cells contain discrete nucleoids spread along the length of the cell, whereas others have large clumps of staining material and large DNA free areas. Blakely *et al.*, (1991) show that *xerC* mutants display a very similar phenotype. In addition, they record that *xerC* mutant cultures form small colonies when growing on solid media and recover slowly from stationary phase. They also note that the filamentous phenotype of these mutants is very variable in its severity.

3.4 Methods used for microscopic analysis

The description of these phenotypes of *dif* and *xerC* mutants, and the photographs shown appear very similar to each other. Therefore, phenotypic comparisons were carried out with these, and mutants of the newly discovered *xerD* gene. It was important to know whether the phenotypes were identical in their characteristics and severity, or whether differences might shed light on the biological role of each component in the recombination system. Therefore wild-type and mutant strains were extensively examined by light microscopy. To facilitate the observation of differences between wild-type and mutant cultures, all cultures were grown in either of two ways.

i. Cultures grown in liquid media were initially very dilute (e.g.. 10 µls of stationary-phase culture, sub-cultured into 20 mls of fresh broth) and subsequently grown for approximately 5 hours. Thus, at examination, cultures were still in exponential-phase, but had been growing long enough for long filaments to form (long filaments are generally absent from *dif* or *xer* mutant stationary-phase liquid cultures).

ii. Colonies of cells were taken from media-containing plates after overnight growth (e.g.. 15 hours). With mutant strains, these cell populations were found to contain large numbers of very long filaments. Since colonies are still increasing in size at this stage of growth, these cells may not be in true stationary-phase, avoiding the corresponding loss of filaments seen in liquid cultures.

A number of different methods were used in the preparation of cells for microscopic analysis.

Untreated live cultures in liquid media. Cultures were grown in LB broth as described, 10 µl samples were placed on slides, and observed untreated under a cover slip. Samples were viewed under phase-contrast, or using Nomarski optics.

This method avoids the use of preparatory treatments that might interfere with the cell length distribution of a culture (e.g.. washing fixed cells might be expected to wash more short cells off the microscope slide than filaments). As cells are still active, motile and not fixed onto the microscope slide when viewed, this technique has the disadvantage that photographs of these cultures are of poor quality due to a lack of depth of field and motion of the cells during the necessarily long exposure times.

Unfixed cells adhered to slides. 1 ml samples of cultures growing in liquid media were harvested at 2,000 rpm for 10 minutes, re-suspended in 1 ml 0.84% NaCl, and 10 µl samples allowed to dry onto microscope slides. Cells were then stuck onto the slide surface using 10 µl of poly-L-lysine slide adhesive solution, and allowed to dry. 10 µl of 0.84% NaCl and a coverslip were added, before observation using Nomarski optics. This technique has the advantage that preparations are well suited to photography, with a minimum of possible interference with the cell length distribution of cultures. Centrifugation of cells at speeds up to 15,000 rpm has no visible effect on the cell length distribution seen.

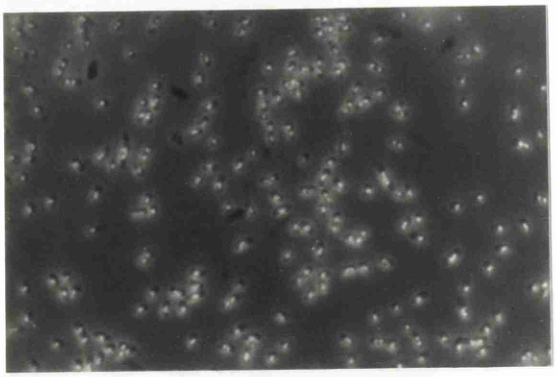
Staining of cell nucleoids with the fluorescent stain DAPI. This method, adapted from Hiraga *et al.*, (1989) and Eliasson *et al.*, (1992) allows visualisation of cell nucleoids by use of the fluorescent dye 4', 6-diamidino-2-phenylindole (DAPI) that selectively binds into DNA. Cells were washed, fixed with cold methanol, washed again and stuck onto slides, before addition of 1 µg/ml of DAPI in 70% iso-tonic Saline / 30% glycerol. Cells were viewed under phase contrast with UV illumination.

3.5 Comparative analysis of the *dif/xer* phenotype

Microscopic analysis showed that the two *dif* strains created, NL40 and NL41, and the *xerD* strains DS9008 and STL116 demonstrate this filamentous morphology (Fig 3.7 and data not shown). These strains also produce smaller colonies than isogenic wild-type strains when grown on solid media. When mutant strains were directly compared, extensive microscopic analysis of the morphology of the mutant strains NL40(*dif*), DS984(*xerC*) and DS9008(*xerD*), amongst others, using all of the preparatory methods described above showed them to be indistinguishable from each other (Fig. 3.7 and data not shown). DS984 and DS9008 carry transposon insertion mutations and do not support any detectable Xer recombination between *dif* or *cer* sites on plasmids (Colloms *et al.*, 1990; Blakely *et al.*, 1993). The positions of these transposon insertions in *xerC* and *xerD* correspond to positions approximately 100 amino acids, and 240 amino acids respectively into the corresponding proteins from the amino termini. Both of these proteins are 299 amino acids long, and have their most conserved sequences, believed to contribute to their active site, close to the carboxy termini. Therefore, although peptides may be formed in these strains from mutated genes, they are null mutants with respect to recombination. Strains containing mutations in both *xerC* and *xerD* (DS9009), *xerC* and *dif* (NL50), and *xerD* and *dif* (NL45) were also produced. Phenotypic analysis of untreated cultures of these double mutants found them to exhibit a phenotype indistinguishable from that of single mutants (data not shown).

It was also important to better understand the effects, if any, of filamentation upon growth and viability of mutant cultures. As described above, the number of filaments in mutant cultures when compared to wild-type is very variable, being most easily visible in cultures that have been growing for several hours without entering stationary phase. For these reasons it was decided to analyse the growth of wild-type and mutant strains by recording the optical

A



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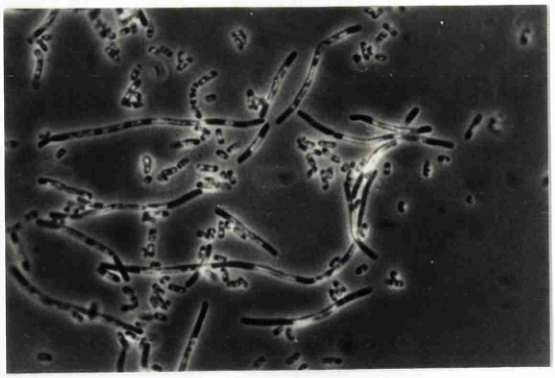

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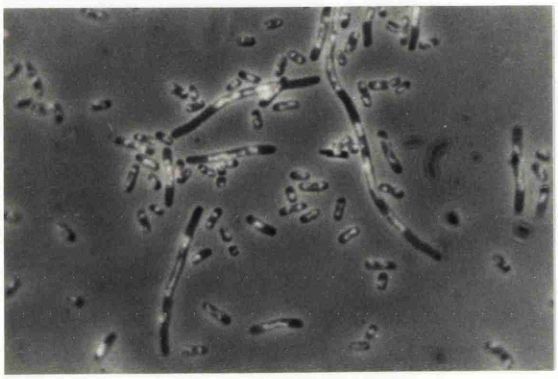

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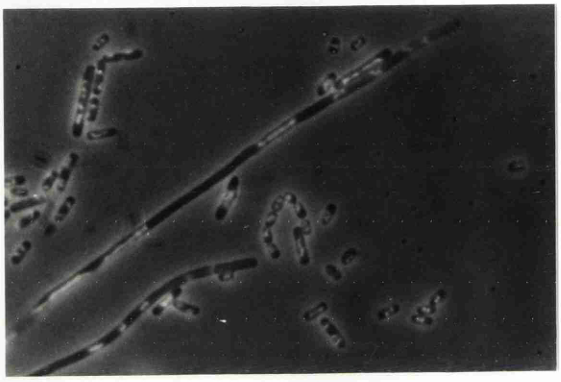

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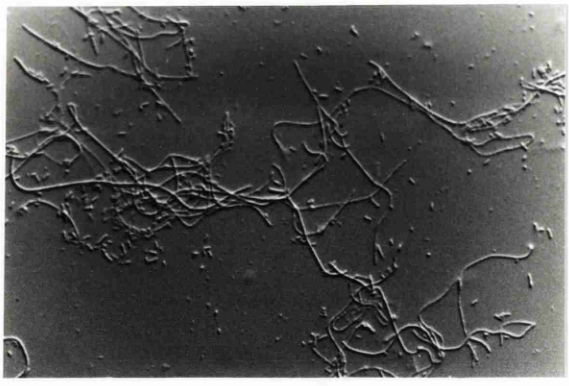

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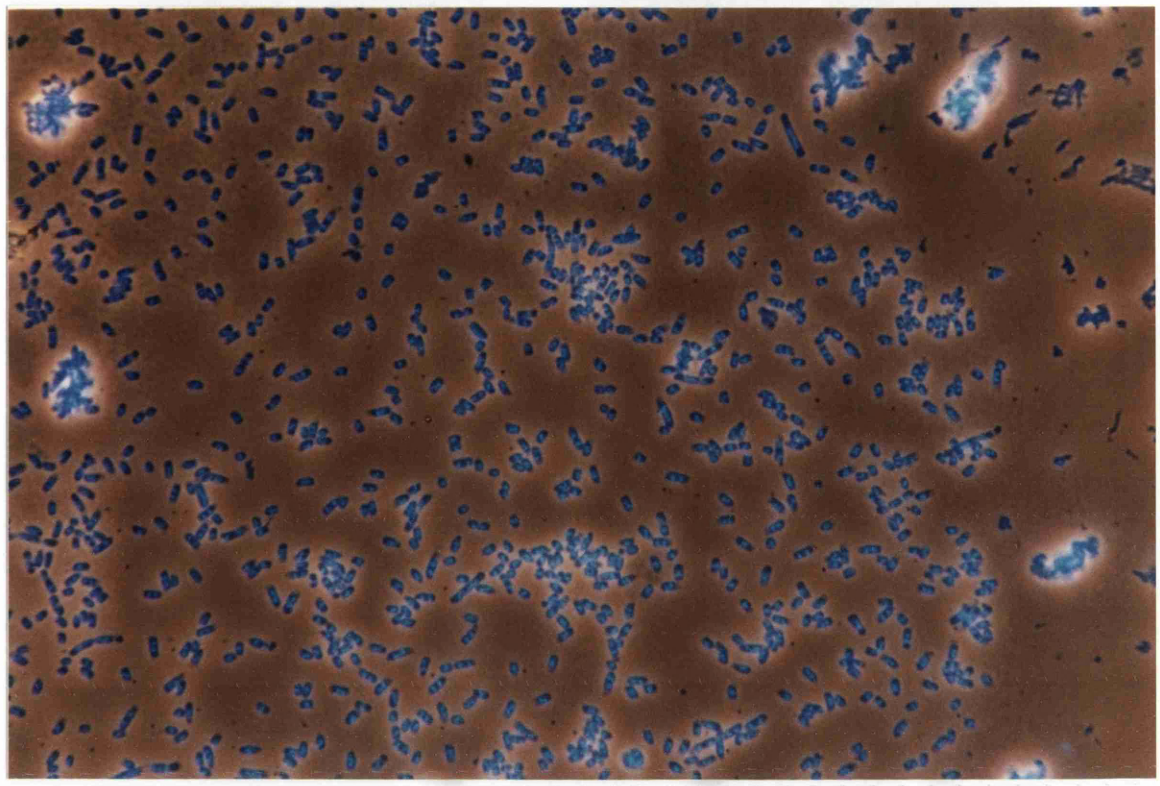


B'

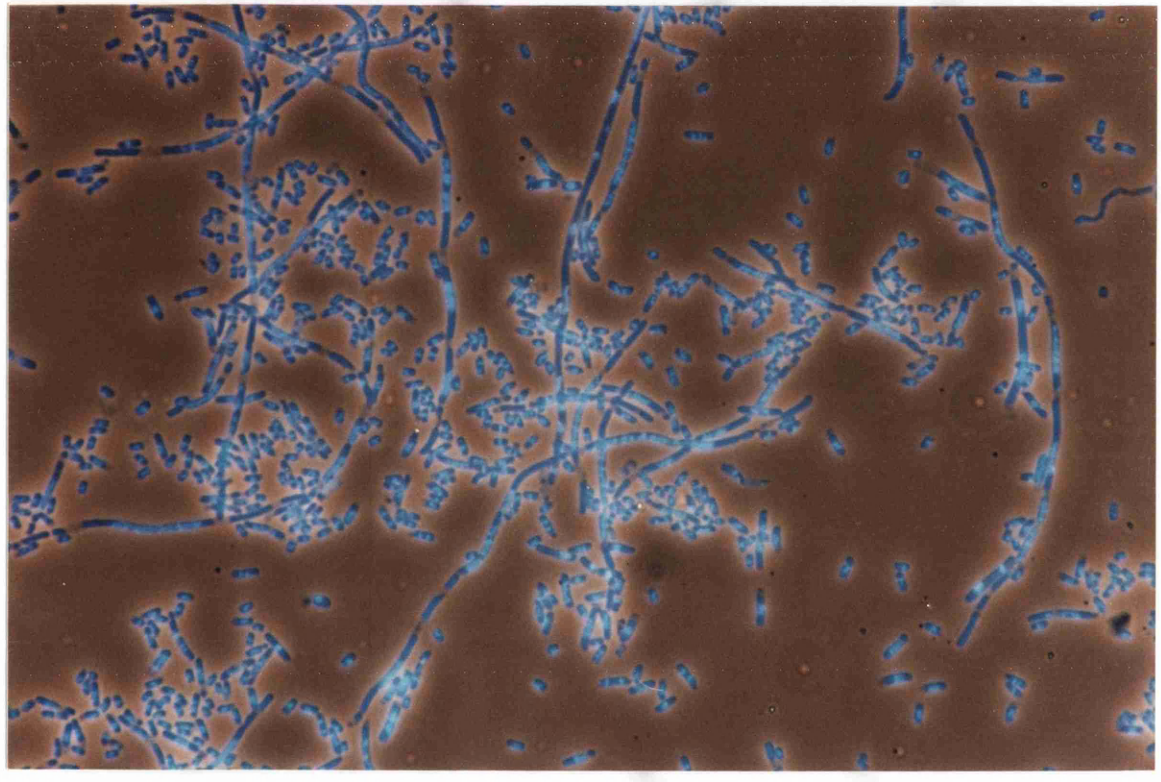


Figure 3.7. The cell and nucleoid morphology of *dif* and *xer* mutants. Exponential phase cultures grown in LB broth at 37° were photographed untreated, adhered to slides (photographs E and F), or after staining of the nucleoid material with DAPI (photographs A, B, C and D). These methods are described in the main text and in Materials and Methods. (A) DS941 - wild-type. (B) NL40 - *dif*. (C) DS984 - *xerC*. (D) DS9008 - *xerD*. (E) DS941 - wild-type. (F) NL40 - *dif*. The long axis of each photograph represents approximately 70 μm (A-D), 140μm (A' and B') or 280 μm (E and F).

density and viable cell count of liquid batch cultures at different stages of growth.

Analysis of growth rate and viable cell count was performed upon cultures of DS941 (wild-type), DS984 (*xerC*), DS9008 (*xerD*) and NL40 (*dif*). Cultures were grown in 100ml volumes of LB broth in 500ml conical flasks at 37° and shaken at approximately 200 rpm. Duplicate samples were removed initially every 15 minutes, and later at less frequent intervals, for the determination of optical density at a wavelength of 600nm. Times taken for cultures to double in OD, and the duration of lag phase, were calculated by the methods described in Niedhardt *et al.*, (1990). The calculated doubling times, a plot of OD against time, and the viable cell counts at various times are presented in Table 3.2, Figure 3.8 and Table 3.3 respectively.

The data show a repeatable difference in the length of lag phase (data not shown) and the doubling time between wild-type cultures and the three mutants, both being longer for mutants. They show no repeatable difference between the three types of mutant however. The longer lag phase of mutant cultures relative to the wild-type could be caused by reduced viable cell counts per OD unit in the culture at inoculation. Determination of the viable cell counts of the mutant stationary phase cultures used for this inoculation consistently show reduced viability relative to wild-type by a factor of approximately two (data not shown). A real difference in the speed of metabolic adaptation to fresh rich media between wild-type and mutant cells seems unlikely, and has not been seen in other experiments in which the viability of the cultures at inoculation was controlled (data not shown). The lower observed growth rates of mutant cultures could be artefactual if filamentous cells contributed significantly less to measured OD per unit mass than other cells and were making up a significant proportion of the cells in the cultures investigated. However, this seems unlikely, as early exponential phase cultures, from which these doubling times are calculated contain few visible long filaments.

Strain	DS941	DS984	DS9008	NL40
	wild-type	*xerC*	*xerD*	*dif*
Doubling times in minutes	22.5	26.5	25.5	28
	23	24.5	26	26.5
	22.5	24.5	25	25
	23	23	25.5	23
	23	25.5	26.25	26.5
	22.75			
	23.5			
Mean	**22.9**	**24.8**	**25.65**	**25.8**
Standard Deviation	**0.35**	**1.30**	**0.49**	**1.89**

Table 3.2. Doubling times of wild-type and *dif/xer* mutant cultures during exponential growth. Each item represents one independent calculation of the doubling time of these strains during exponential growth phase in a batch culture under the same conditions. Also included are the calculated mean ($\bar{x}$) and standard deviation ($\bar{x}$sn-1) for each set of results.

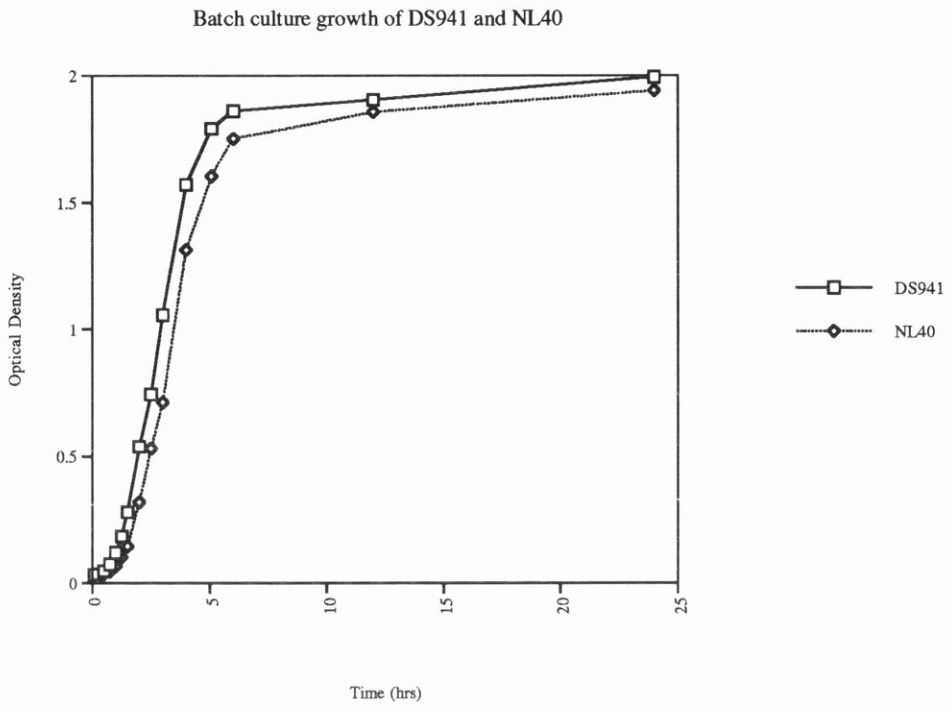


Figure 3.8. Growth of DS941 (wild-type) and NL40 (*dif*) in batch culture. OD^{600} was plotted against time for cultures growing in 100 mls LB broth in 500 ml conical flasks at 37° with rapid shaking. Initial inoculation used 1ml of staionary phase culture. Each data point is plotted as the average OD of two duplicate culture samples. The data presented are single representative examples of wild-type and mutant growth curves. The measured maximum stable optical density of NL40 and other mutant cultures in stationary phase was not consistently lower than that of DS941 in all experiments. The relationship between time and viable cell count in these experiments is shown in Fig. 3.10.

	Strain			
	DS941	DS984	DS9008	NL40
Time (hrs)				
3 hrs	59 45 48 51 40	23 29 12	30 25 11	31 23 26
mean ($\bar{x}$) S.D ($\bar{x}$sn-1)	**48.6** **7.1**	**21.3** **8.6**	**22** **9.8**	**26.7** **4.0**
6 hrs	171 166 150 123 119 123 136	86 88 75 59 76	100 60 79 56 56	83 38 53 46 58
mean ($\bar{x}$) S.D ($\bar{x}$sn-1)	**141.1** **21.5**	**76.8** **11.5**	**70.2** **19.2**	**55.6** **17.1**
12 hrs	134 153 126 97 129 162 168	167 128 123 120 150	124 107 112 108 115	155 126 125 111 128
mean ($\bar{x}$) S.D ($\bar{x}$sn-1)	**138.4** **24.6**	**137.6** **20.2**	**113.2** **6.8**	**129.0** **16.0**
24 hrs	92 80 92 70 80	101 7 † 87	76 157 73	88 103 100
mean ($\bar{x}$) S.D ($\bar{x}$sn-1)	**82.8** **9.3**	**65 †** **50.7**	**102.0** **47.7**	**97.0** **7.9**

Table 3.3. Variation of viable cell counts per OD unit with time during batch culture growth. Each entry in the table represents one independant calculation of the number of viable cell counts per unit of optical density, at various time points during batch culture growth. The mean and strandard deviation of each set of results are also presented. Units are (colonies $*10^7$) / ml / Abs^{600}. Most entries are calculated from the mean colony count from duplicate or triplicate platings of the same diluted culture. The relationship between time and growth phase in these experiments is seen in Fig. 3.9. † - The mean and standard deviation of the 24 hr results for DS984 are greatly affected by the marked result of 7. This result may have been caused by a mistake during dilution of the initial culture (eg incomplete mixing), and should not be taken to indicate a significant indication of a variation in viability.

There is also a difference in viable cell counts per OD unit between the wild-type and mutant cultures at some stages of growth (Fig. 3.8 and Table 3.3). After 3 hours and 6 hours of growth, while these cultures are in exponential phase or just entering stationary phase, wild-type cultures consistently contain more viable cells per OD unit than mutants, by a factor of approximately two. This difference is not evident after 12 or 24 hours, in cultures that have been in stationary phase for some hours. One obvious explanation for a drop in viable cell count per OD unit in mutant cultures is the appearance of filamentous cells within the culture. Filaments would be expected to significantly over-contribute to the optical density of a culture but to contribute no more than any other cell to the viable cell count. Indeed, microscopic observation of *dif* mutant filaments growing on solid media supports the idea that they are unable to divide again once initial filamentation has occurred (Peter Kuempel, personal communication). If this is the case, filaments are a dead-end with respect to colony formation, and would not contribute to the viable cell count. This hypothesis that a drop in viable cell count per OD unit is caused by the presence of filaments within a culture is supported by the finding that microscopic analysis of liquid cultures shows much greater similarity between wild-type and mutant cultures during stationary phase than in exponential phase. The viable cell count per OD unit also shows greater similarity between wild-type and mutant cultures during stationary phase.

The increase in doubling time seen with mutant cultures could also be explained by the appearance of filaments, as a filamentous cell might not be able to grow exponentially. Once a filament has formed, the induction of the SOS response and possibly the decrease in the number of cell ends per unit mass, and the production of large DNA free areas within the cell might interfere with cell growth. This in turn would lead to a longer measured doubling time of the filamentous culture. It could be argued that a growth rate difference between wild-type and mutant cultures could be caused by the expression of proteins

detrimental to cellular metabolism from the antibiotic resistance genes contained in all three mutants, but not in wild-type. However, long term constant growth analysis using a chemostat does not show any significant growth rate difference between isogenic *E. coli* strains with and without chromosomal insertions of the kanamycin resistance gene from Tn*5* (Biel *et al.*, 1983). Since this gene is very closely related to the resistance gene from Tn*903* used here (Oka *et al.*, 1981; Beck *et al.*, 1982), the significant growth rate difference seen in our experiments should not be explained on these grounds.

Under casual microscopic observation, healthy cultures of wild-type and *dif* or *xer* mutant cultures appear to be clearly different in their morphology (Fig 3.7). However, how much of this effect is caused by a few very long filaments, and whether there is a very significant difference between the cell length distributions of wild-type, *dif*, *xerC* and *xerD* mutants, is harder to determine. Therefore, statistical analysis was performed upon the cell length distributions of cultures. Cultures of DS941, DS984 and NL40 were grown at 37° for several hours into late exponential phase in LB broth and photographed untreated using Nomarski optics. Slides were projected onto a wall and cell lengths measured for 167 cells of each culture of DS941 and NL40, and 233 cells of DS984. The mean and standard error of the mean of the three populations are presented in Table 3.4. Both DS984 (*xerC*) and NL40 (*dif*) give results demonstrating a very significant difference from wild-type. These results strongly support the assessment of cultures made by simple microscopic observation.

By microscopic observation, and analysis of cell lengths, growth rates and viable cell counts of mutant cultures, the phenotypes caused by presumptive null mutations in *dif*, *xerC*, or *xerD* are indistinguishable, and characteristic. Considering the known interactions of XerC and XerD proteins with *dif* (Blakely *et al.*, 1991; Blakely *et al.*, 1993) this seems very strong evidence that the phenotype is caused by the abolition of a particular biological function provided by a system requiring these three components.

Strain	Number of cell lengths counted	Mean Cell length	Standard error of the mean
DS941 (wt)	167	2.71 μm	+/- 0.07 μm
DS984 (*xerC*)	233	3.96 μm	+/- 0.39 μm
NL40 (*dif*)	167	3.74 μm	+/- 0.24 μm

Table 3.4. Analysis of cell length distribution. The difference in cell length distribution between wild-type and mutant exponential phase cultures was analysed. These data were obtained by measuring 167 cell lengths each from DS941 (wild-type) and NL40 (*dif*), and 233 from DS984 (*xerC*). The mean ($\bar{x}$), and strandard error of the mean ($\bar{x}sn-1/\sqrt{N}$) were calculated. These findings agree well with previous work on individual mutants (Kuempel *et al.*, 1991; Blakely *et al.*, 1991).

3.6 The *recF* mutation of DS941 does not affect the *dif/xer* phenotype

Mutations in *recF* greatly reduce inter-plasmid homologous recombination (Cohen and Laban, 1983). This allows easier analysis of inter-plasmid site-specific recombination in *in vivo* recombination assays. For this reason, and because most *xerC* and *xerD* strains constructed share this genetic background, it was decided to conduct the majority of our experiments in *recF* strains based upon DS941 (AB1157 *recF143*, *lacZΔM15*, *lacI*^{q}). However, mutations in some recombination genes (e.g.. *recA* or *recBC*) are known to affect cell morphology (Chaudhury and Smith, 1984; Zyskind *et al.*, 1992), and appear to suppress the *dif* phenotype (Kuempel *et al.*, 1991). Although it is clear that the *recF* mutation in DS941 alone does not cause a morphological phenotype (Fig. 3.7), it was important to know that it does not alter the phenotype caused by mutations in *dif* or the *xer* genes. Therefore, the largest *dif* deletion, *difΔ6*, and the *xerC::y17* allele were each transduced into recombination proficient AB1157 to give NL80 and NL90 respectively. Phenotypic analysis of untreated cultures of these, and corresponding *recF*$^{-}$ strains grown in LB broth at 37° failed to show any difference in the phenotype expressed between *recF*$^{-}$ and *recF*$^{+}$ strains (data not shown).

Chapter 4

Functional analysis of *dif* site-specific recombination

Only three kinds of genetic rearrangement can result from productive recombination between two correctly aligned site-specific recombination sites, fusion (or integration), resolution (or excision) or inversion. This is discussed in the introduction, and shown in Fig. 1.1. With respect to which of these reactions are catalysed, recombination at all well characterised natural site-specific recombination sites falls into one of two groups, here called Unconstrained, or Selective.

Unconstrained recombination systems. Unconstrained recombination systems will catalyse reactions between sites in any topological configuration, resulting in fusion, resolution or inversion. These recombination systems appear to be simple; recombination *in vitro* requiring only a core recombination site without accessory sequences, and recombinase enzymes (Sternberg *et al.*, 1983; Gronostajski and Sadowski, 1985a). Two such systems have been extensively studied *in vitro*, the *loxP*/Cre system of bacteriophage P1, and the *frt*/FLP system of the yeast 2 μm plasmid (Cox, 1989; Hoess and Abremski, 1990). Although *FRT* naturally contains an extra FLP binding site adjacent to the normal core recombination site, this extra 13 bp sequence is not required for recombination (Gronostajski and Sadowski, 1985a, Andrews *et al.*, 1987). Both use recombinase enzymes of the lambda integrase family (Chapter 1.5), as do all other naturally occurring unconstrained recombination systems. However, unconstrained recombination is not a phenomenon mechanistically restricted to the integrase family of recombinases, as a single amino acid substitution mutation within the Gin invertase of bacteriophage Mu can change Gin recombination from a selective system to an unconstrained system. This Gin mutation makes

recombination independent of the accessory protein FIS and the recombinational enhancer sequence *sis* (Klippel *et al.*, 1988; Crisona *et al.*, 1994).

Selective recombination systems. Selective systems will only catalyse reactions between sites in a particular topological configuration, e.g. the *res*/resolvase system of transposon Tn*3* will only catalyse resolution reactions between two *res* sites in direct repeat in the same DNA molecule; sites in other configurations do not react efficiently. In order to enforce this selectivity, larger, more complex sites, and often other proteins, are required than with unconstrained systems. Examples of such systems include *cer*/Xer from plasmid ColE1, *res*/resolvase from Tn*3*, *att*/integrase from bacteriophage λ and *gix*/Gin from bacteriophage Mu. The recombination site substrates in these systems are invariably more complex than simply a core recombination site. Core sites are bound by recombinase enzymes and contain the sites of strand exchange. However, in selective systems these core sites are not sufficient substrates for recombination, but require additional flanking sequences (e.g. 100-200 bp) that are necessary for recombination to occur. These additional or accessory sequences contain protein binding sites, usually for extra proteins required in addition to the recombinase. There is strong evidence from several systems that neither accessory proteins nor sequences are intimately involved in the catalysis of strand exchange, but that they hold core sites together in order that a productive recombinational synapse can be formed, and are involved in enforcing selectivity (Craig and Nash, 1983, Klippel *et al.*, 1988b, Landy, 1989; Summers, 1989; Stark and Boocock, 1995).

The mechanism by which these extra proteins and sequences regulate recombination is the subject of ongoing work. It is believed that protein-DNA and protein-protein interactions between accessory proteins, accessory sequences and recombinase enzymes of recombining sites hold the DNA flanking the point of recombination in a particular configuration, and only pairs of recombination

sites synapsed together in this configuration will undergo productive recombination. By thus controlling the geometrical configuration of the DNA immediately surrounding the site of recombination, only for sites in the correct topological arrangement might recombination be energetically favourable.

The λ *att*/integrase recombination system (reviewed by Landy, 1989 and described in Chapter 1.5) has been very well characterised *in vitro* and *in vivo*, displaying the characteristics of a complex selective system very well. It recombines large, non-identical sites (totalling ≈270 bp) containing multiple protein binding sites for four different proteins, the recombinase Int, as well as the DNA binding proteins, IHF, FIS and Xis. The core site, to which Int binds, is not a sufficient substrate for recombination without flanking sequences and accessory proteins. The control imposed by the accessory sequences and proteins is particularly clear in this system *in vitro*, as the reactions catalysed at the site (integration vs excision) are variable, depending upon the relative concentrations of the accessory proteins in the reaction, particularly Xis and FIS. In this system the determination of the relative configuration of recombining sites is facilitated by the non-identical nature these sites.

4.1 The possible complexity of the *dif* site

A 33 bp DNA fragment containing the 28 bp *dif* core has been shown to be a sufficient substrate for Xer-mediated multimerisation and resolution of plasmids *in vivo*, when cloned into the high copy-number vector, pUC18 (Blakely *et al.*, 1991). *In vitro* this fragment binds XerC and XerD and can support a partial recombination reaction (Blakely *et al.*, 1993, G. Blakely personal communication). Since *dif* has a core site that is a sufficient substrate for unconstrained recombination, this might suggest that *dif* was part of a simple unconstrained recombination system, like *loxP*/Cre and *frt*/FLP. However, Summers (1989)

describes an artificial complex recombination site acted upon by XerC and XerD, the type II hybrid or *cer6* site, that displays both recombination selectivity and a core site that will undergo recombination in the absence of accessory sequences and proteins. *cer6* is a hybrid site created by Xer-mediated recombination between *cer* of plasmid ColE1 and *parB* of the related plasmid CloDF13 (described further in Chapter 5). If the accessory sequences of this hybrid are deleted, its core site has the same unconstrained substrate properties as the core site of *dif* used by Blakely *et al.*, (1991). However, the full site including accessory sequences (≈250 bp) displays some resolution selectivity in the presence of the accessory proteins ArgR and PepA, preferentially recombining sites found in direct repeat within the same molecule.

Is the same true for *dif*? Does *dif* too have accessory sequences and proteins that can regulate the recombination occurring at its core site? All well characterised site-specific recombination systems that naturally recombine sites in the same molecule, cause reaction between sites separated by only a few kilobases of intervening DNA. It seems unlikely that a mechanism able to enforce resolution selectivity upon sites separated by a few kilobases of DNA would be unaffected by a separation of 4.7 Mb, the size of the *E.coli* chromosome (Stark and Boocock, 1995). However, a mechanism ensuring chromosomal monomerisation through resolution selectivity, although possibly of a very different nature to the mechanisms employed in well studied site-specific recombination systems, might well involve extra proteins binding to sequences close to the *dif* core site. Thus experiments were undertaken to investigate these possibilities.

4.2 Does Lrp play a role in Xer recombination?

Blomfield *et al.*, (1993) show that Lrp, the leucine-responsive regulatory protein, plays an important role in the site-specific inversion catalysed by FimB

and FimE in type-1 fimbrial phase variation in *E. coli*. FimB and FimE are amongst the closest natural homologues to XerC and XerD, and also act in one of the very few other site-specific recombination systems utilising two recombinases. Results indicated that the role of Lrp was in the actual recombination mechanism itself, rather than in the control of transcription of another component of the system. When computer generated alignments of these proteins were prepared, these generally revealed approximately 25% identity and approximately 50% similarity between the amino acid sequences of each Xer protein when compared to each Fim protein. This compares to 37% and 50% identity respectively between the two Xer proteins and the two Fim proteins themselves. Therefore, it seemed possible that Lrp might be involved in recombination at *dif*. This possibility was investigated by analysing *dif*/Xer recombination *in vivo* in strains mutant for *lrp* (also known as *mbf*), the gene encoding Lrp.

These *in vivo* recombination assays were performed using 'reporter plasmids'. The reporter plasmids used in this work are plasmids containing selectable marker genes, flanked by directly repeated copies of Xer-dependent site-specific recombination sites, including *dif*. In order to detect inversion reactions, inverted sites could be used. The reporter plasmid pKS455 is used in this work, and shown as an example (Fig. 3.1; Stirling *et al.*, 1988a). This plasmid, based upon pUC9, contains two directly repeated *cer* sites flanking a chloramphenicol resistance gene. If transformed into an Xer^+ strain, recombination between the *cer* sites will delete the resistance gene, giving a replicative ampicillin resistant chloramphenicol sensitive plasmid, pKS456, and a non-replicative circle pKS457. This deletion event can be detected by scoring for chloramphenicol resistance or by analysing DNA by gel electrophoresis. Here, three reporter plasmids are used, pSD124, pKS455 and p752, each containing directly repeated copies of the Xer recombination sites *dif*, *cer*, and *psi* respectively.

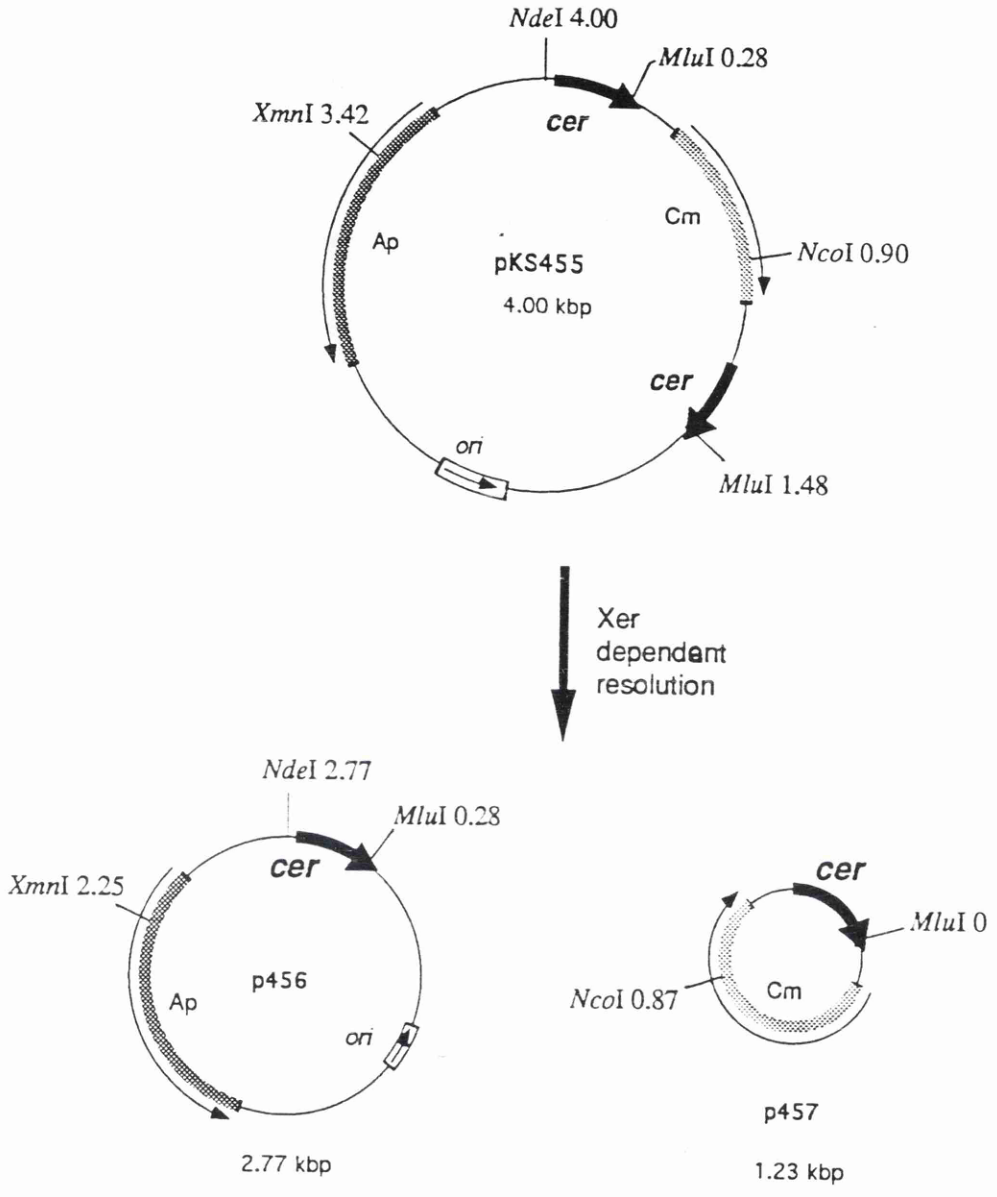


Fig. 4.1. The *cer* reporter plasmid pKS455. pKS455 is a pUC9 based reporter plasmid containing two directly repeated *cer* sites, flanking a chloramphenicol resistance gene (Stirling *et al.*, 1988a). The plasmid is resolved *in vivo* in *xer*$^+$ strains to give pKS456 (2.77 kb) and pKS 457 (1.23 kb). As only pKS456 contains a replication origin, it is generally the only product detected *in vivo* in recombination assays. In this diagramatic representation, the ampicilin and chloramphenicol resistance genes, the two *cer* sites and the origin of replication are represented. This diagram is adapted from McCulloch, (1992).

Two different mutant alleles of *lrp*, both marked by a tetracycline resistance gene were kindly provided by David Low, *mbf (lrp)-18* and *mbf (lrp)-20* (Braaten *et al.*, 1992) in the strains DL842 and DL844 respectively. However, these strains were fully proficient for homologous recombination, and therefore unsuitable as hosts for reporter plasmid analysis. Therefore bacteriophage P1 transduction was used to transfer these alleles into DS941 to give strains NL60 (DS941 *mbf (lrp)-18*) and NL70 (DS941 *mbf (lrp)-20*). Both *lrp* strains form smaller colonies than DS941 on Minimal Medium supplemented with 0.4% Glucose, or Minimal Medium supplemented with Casamino acids (Materials and Methods), but not on L-B medium, as noted by Ernsting *et al.*, (1992). DS941, NL60, NL70 and DS984 (DS941 *xerC*) were then transformed with the three reporter plasmids pSD124, pKS455 and p752, looking at Xer-dependent recombination at *dif*, *cer* (from plasmid ColE1), and *psi* (from plasmid pSC101) respectively. Ampicillin resistant transformant colonies were isolated and grown overnight before plasmid DNA was prepared by the single colony lysis method and analysed by agarose gel electrophoresis and ethidium bromide staining. No deficiency in Xer recombination was evident in NL60 or NL70, as in all cases plasmid DNA was indistinguishable from that prepared from DS941 (Fig. 4.2 and data not shown).

Therefore, Lrp is not required for Xer-dependent recombination at *dif*, *cer*, or *psi*, and plays no detectable role in these recombination events, as analysed by this *in vivo* plasmid assay. This result is in accordance with more recent work by Gally *et al.*, (1993) looking at environmental regulation of the *fim* switch. They show that the role of Lrp in *fim* catalysed switching allows this function to respond to the composition of the external medium. They suggest that this could allow differential expression of type-1 fimbriae in different host compartments or in an external environment. This agrees with the hypothesis that the role of Lrp as a global regulator in *E. coli* is to switch gene expression between states required in a host and in an external environment (Newman *et al.*, 1992).

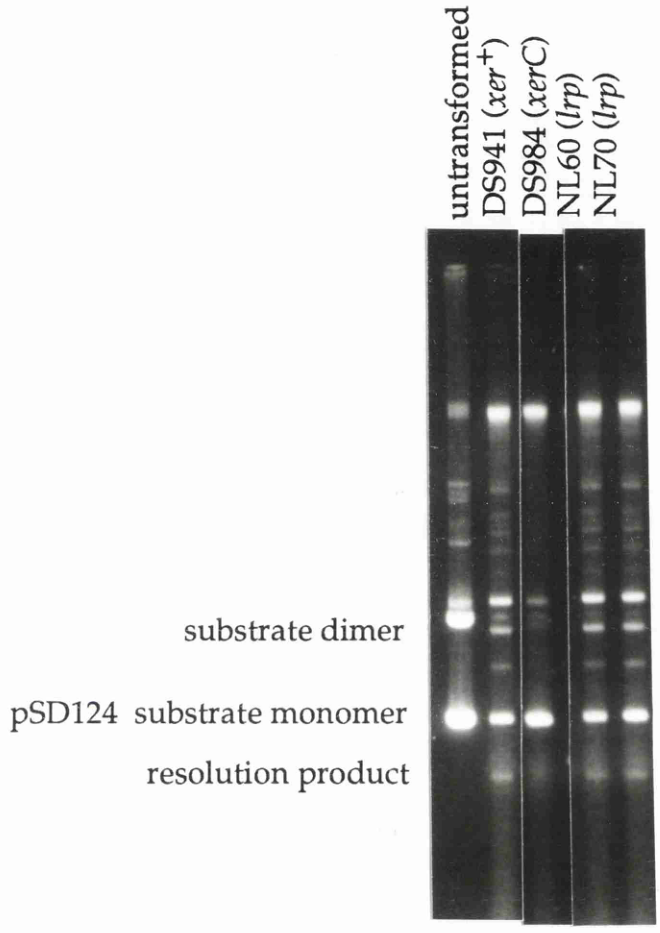


Fig. 4.2a. Mutations in *lrp* have no effect on Xer recombination. (A) The reporter plasmid pSD124, containing two directly repeated *dif* sites, was used to transform DS941 (*xer*$^+$), DS984 (*xerC*), NL60 (*lrp*) and NL70 (*lrp*). DNA was prepared and analysed by agarose gel electrophoresis, with untransformed plasmid DNA loaded as a control, giving the result above. The pattern of recombination in NL60 and NL70 is not significantly different from that in DS941. Therefore, the *lrp* mutation in these strains appears to have no effect upon the Xer recombination seen in this assay.

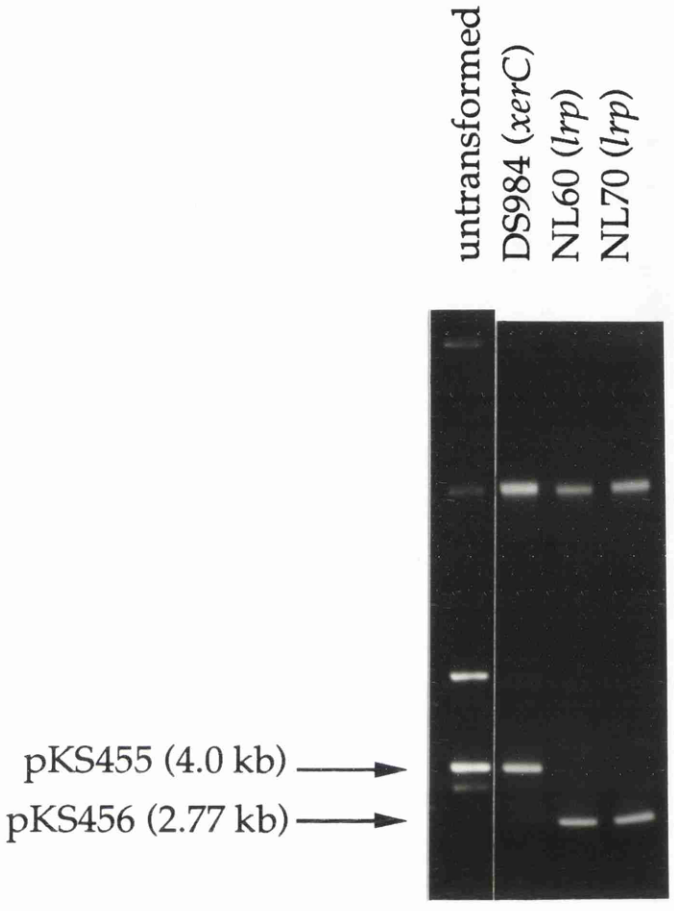


Figure 4.2b. Mutations in *lrp* have no effect on Xer recombination. (B) The reporter plasmid pKS455 (Fig. 4.1), containing two directly repeated *cer* sites, was used to transform DS984 (*xerC*), NL60 (*lrp*) and NL70 (*lrp*). DNA was prepared and analysed by agarose gel electrophoresis, with untransformed plasmid DNA loaded as a control, giving the result above. The breakdown of pKS455 to pKS456 in NL60 and NL70 is not significantly different from that in DS941. Therefore, the *lrp* mutations in these strains appears to have no effect upon the Xer recombination seen in this assay.

4.3 Chromosomal flanking sequences have no detectable effect on *in vivo* recombination at *dif* in a plasmid substrate

As previously discussed, there is good evidence that in many recombination systems, sequences flanking a core recombination site impose selectivity on the recombination catalysed there. If chromosomal sequences flanking the *dif* core site play a role in the recombination catalysed there, sites with and without these sequences would be expected to display different properties in recombination assays when inserted in plasmids. Kuempel *et al.*, (1991) suggest such a difference, but exact details of their work are unclear. There is good evidence that a 532 bp chromosomal fragment containing the *dif* core site carries all the sequences required for the wild-type functioning of *dif* (Kuempel *et al.*, 1991). Therefore, we used an *in vivo* recombination assay to compare the Xer-dependent multimerisation and resolution of plasmids with and without these extra sequences flanking the core *dif* site. Three plasmids were used containing *dif* fragments of 33 bp, 247 bp and 532 bp (Fig. 4.3a). Each plasmid consists of a *dif* fragment inserted into the polylinker of cloning vector pUC18, giving pMIN33, pN30 and pN532 respectively. Plasmids pUC18 and pKS492 were used as controls. pKS492 is pUC18 containing a full length *cer* site in the polylinker. *cer* is an Xer-catalysed recombination site from plasmid ColE1 that displays resolution selectivity (Summers and Sherratt, 1984). Either *recF* or *recA* strains deficient in plasmid homologous recombination were transformed with monomeric or dimeric plasmid DNA purified from agarose gels. Plasmid DNA from transformed strains was prepared after different numbers of generations, from both clonal groups derived from individual transformation events, and from pooled collections of transformants. DNA preparations were analysed by agarose gel electrophoresis and stained with ethidium bromide, for visualisation of the relative fractions of plasmid DNA present in monomeric, dimeric or other higher multimeric form. Repeated experiments did not show any distinguishable

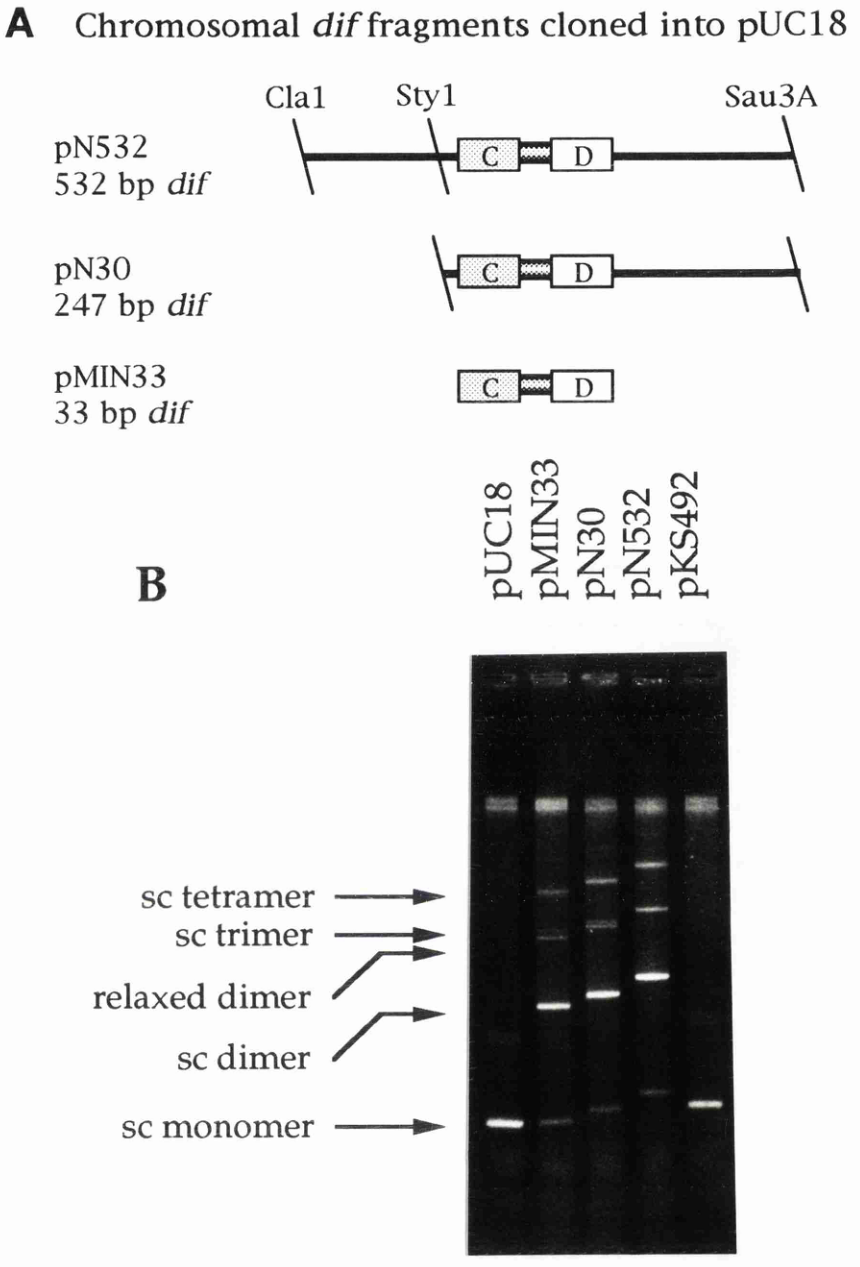


Fig. 4.3. Chromosomal flanking sequences have no detectable effect on *in vivo* recombination at *dif* in a plasmid substrate. (A) The three *dif* containing fragments of sizes 532, 242 and 33 bp cloned into the polylinker of pUC18 are shown. pKS492 is of similar construction but contains a full *cer* site (Summers and Sherratt, 1984). Boxes labelled C or D correspond to the nucleotide sequences bound by XerC and XerD respectively, with the central region between. **(B)** An example of the *in vivo* recombination assays performed. A *recF* strain (DS941) was transformed with purified monomers of each of the five plasmids, pUC18, pMIN33, pN30, pN532 and pKS492. DNA was prepared after 40 hours of growth and analysed by 1.0% agarose gel electrophoresis. Different plasmid forms on the gel, including higher multimeric forms, are labelled, supercoiled being abbreviated to 'sc'.

difference between the fraction of plasmid DNA present as monomers, or multimers, for the three *dif* plasmids tested. An example of one of these experiments is shown in Fig. 4.3b (and in Fig. 4.10). Thus, the extra sequences flanking the *dif* core site have no detectable effect in my tests on the Xer-dependent multimerisation or resolution occurring *in vivo* in plasmid substrates.

These experiments suggest that sequences flanking *dif* have no role in recombination and that *dif* is part of a simple unconstrained recombination system. However, the natural location of *dif* in the chromosomal replication terminus might allow the action of mechanisms of resolution selectivity that would not function in a plasmid substrate, involving, for example, chromosome partition machinery. It might also be the case that recombination is not the only biological requirement of the *dif* locus if chromosome partition is to proceed normally. In order to look at the functioning of *dif* in the chromosome, experiments were performed looking for *dif* sequences able to suppress the *dif* phenotype caused by the 1500 bp *difΔ6* deletion. Would the core-site effect this suppression?

4.4 Are the chromosomal sequences flanking the *dif* core site required for *dif* function *in situ*?

Kuempel *et al.*, (1991), showed that the phenotype caused by a 12 kb deletion of the *dif* region can be suppressed by integration into this deletion of a plasmid containing the 532 bp *Cla*I-*Sau*3A *dif*$^+$ fragment (Fig 4.3a). However, is the core site alone sufficient for function in the chromosome? To investigate this, fragments containing the *dif* core site sequence were re-introduced into the natural location of *dif*, in the chromosome of a strain containing a large *dif* deletion (NL40) to look for phenotypic suppression. Whereas Kuempel *et al.*, (1991) used the integration of *dif*$^+$ plasmids into a deletion of the *dif* site in a

polA^{ts} strain, it was decided to introduce mutations by the homologous replacement method previously described (Chapter 3.1).

Strategy for re-introduction of recombination sites. As before, mutations were constructed on plasmids before crossing into the chromosome by homologous recombination (Fig. 3.1). An antibiotic resistance gene marker was required for selection of recombinants, that would allow construction of target strains and subsequent transformation with various plasmids. This ruled out kanamycin, chloramphenicol, ampicillin or tetracycline resistance genes. The gentamicin resistance marker from *Pseudomonas aeruginosa* was used (Rubens *et al.*, 1979), the plasmid pGM160 (Muth *et al.*, 1989) being used as a source of DNA. This resistance gene was cloned into the *Hin*dIII site in the centre of the large pMTL23 polylinker (Chambers *et al.*, 1988) in both orientations, to give pN10 and pN12. This cloning vector has several advantages over other available vectors for these experiments. Many different recombination site/resistance gene combinations were constructed, facilitated by the large number of unique restriction enzyme sites and their intelligent organisation in the polylinker of pMTL23 (discussed in Chambers *et al.*, 1988). The positioning of sites for *Xho*I and *Cla*I at one end of this polylinker, and a site at the other for *Nae*I, that cleaves leaving a blunt DNA end, greatly facilitated the introduction of fragments in a controlled orientation, into either the *Xho*I and *Nru*I sites, or the *Cla*I and *Nru*I sites of the kanamycin gene of pNΔ6 (the plasmid containing a 1500 bp deletion of the chromosomal *dif* region, Figs. 3.5 and 4.4), as *Nru*I also leaves a blunt DNA end. Therefore, for the re-introduction of *dif*$^{+}$ fragments into the *dif*Δ6 deletion of NL40, site/marker fragments constructed in this polylinker were cloned into the *Xho*I, *Nru*I and *Cla*I sites of pNΔ6, situated close together in the coding sequence of the kanamycin resistance gene used to delete the *dif* region (Fig. 4.4). This gave plasmids with a recombination site and resistance gene flanked on both sides by several kilobases of homology to the natural location of *dif* in the replication terminus. These

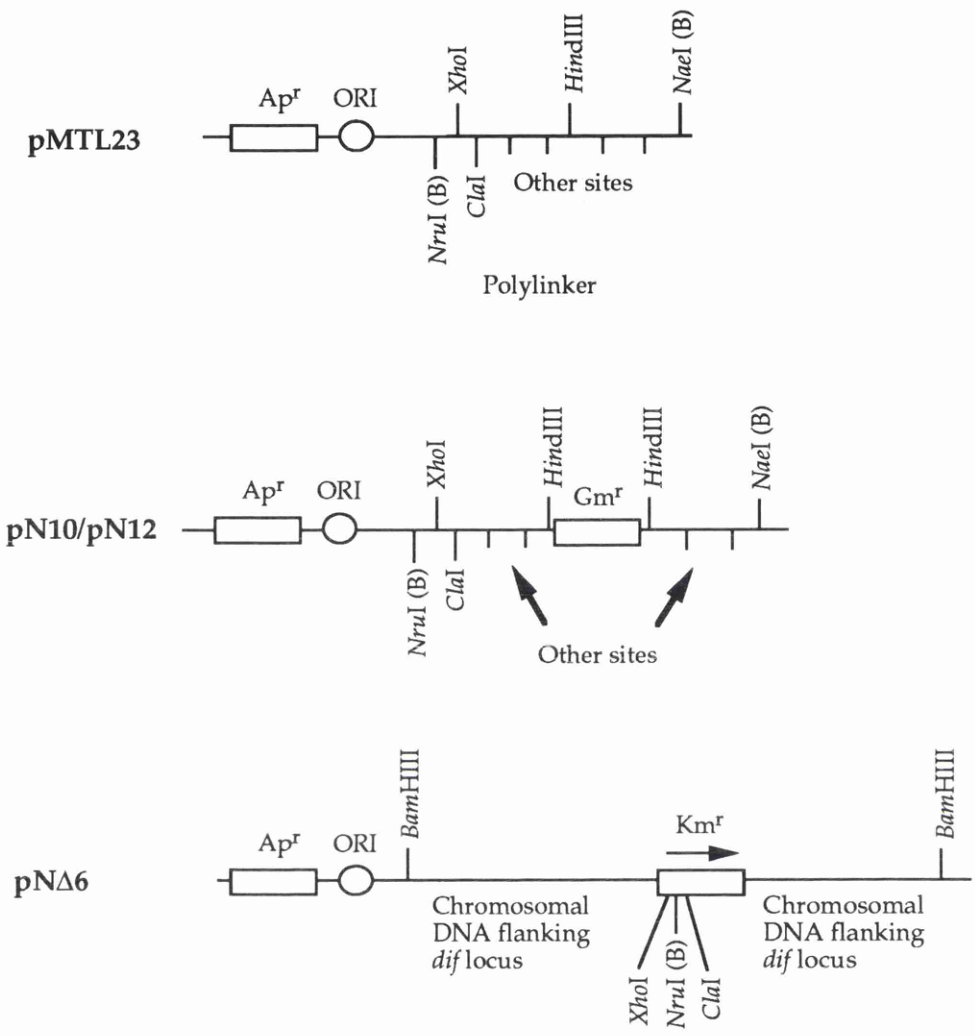


Fig. 4.4. Plasmid strategy for re-introducing recombination sites into *dif* locus DNA. Linear plasmid maps of pMTL23, pN10/pN12, and pNΔ6 are shown. pNΔ6 is also shown in Fig. 3.5. Restriction sites (lines) and antibiotic resistance genes (boxes) are represented, as well as the origin of replication (a circle). Restriction enzyme sites that are cleaved leaving a blunt DNA end are denoted (B). pN10 and pN12 were derived from pMTL23 by insertion of a gentamicin resistance gene into the *Hin*dIII site. pN10 and pN12 are otherwise identical plasmids with the reading frame of the gentamicin resistance gene in different orientations. Recombination sites could be introduced into the labelled 'other sites' of the polylinker in pN10 or pN12, and the restriction sites shown (particularly the *Nae*I site and one of either *Cla*I or *Xho*I) could then be used to clone gentamicin resistance gene/recombination site fragments into pNΔ6 in either orientation, as described in the main text.

plasmids could be linearised and transferred into the chromosome as described earlier (Chapter 3.1). Alleles were first introduced into the *recBC sbcBC* background NL13, which is JC7623 *xerC::y17 difΔ6*, before transduction into a *recF* background. If *dif* locus constructs correctly replaced the *difΔ6* deletion in the chromosome, strains became sensitive to kanamycin, due to the large insertion of sequences into the coding region of the kanamycin resistance gene. Ectopic insertion elsewhere in the chromosome would not have this result. Constructs were always transduced into both DS941 and NL40, two recipient strains that should give almost identical target strains. This allowed a double check of the target strain's phenotype. In all cases, after initial verification of the phenotype by simple microscopic analysis of untreated cultures grown at 37°, only the NL40 based strain was used in further experiments. Transduction into NL40 again caused a change to kanamycin sensitivity. This method for introduction of recombination sites into the chromosome is also used for other recombination sites in later experiments. Strain construction was generally verified by Southern hybridisation.

4.5 Re-introduction of *dif*$^+$ fragments into a deletion of the chromosomal *dif* site.

532 bp and 33 bp *dif*$^+$ fragments (used in plasmid experiments, see Fig. 4.3a) were each introduced into a chromosomal *dif* deletion. The 33 bp *dif*$^+$ core site fragment from pMIN33 was re-introduced into the chromosome in both orientations relative to the surrounding DNA, and in three different positions relative to the reading frames of the kanamycin and gentamicin resistance genes used in construction, giving strains NL286, NL296 and NL246. The 532 bp fragment used by Kuempel *et al.*, (1991) was also re-introduced in both orientations, in two different contexts, giving strains NL247 and NL287. Details

of constructs are shown in Fig 4.5 and Tables 2.1 and 2.2. That sites had indeed been introduced into the correct chromosomal region was verified by hybridisation of an end-labelled *dif* core sequence oligonucleotide to chromosomal DNA cut with *Pvu*II (Fig. 4.6). This showed hybridisation to bands of the size predicted by the physical map of the *E.coli* genome (Kohara *et al.*, 1987). The fragment sizes for some of these strains were also verified in an earlier experiment, showing hybridisation with a restriction fragment from the replication terminus (a radio-labelled *Eco*RI - *Bam*HI restriction fragment, ≈700 bp long, from pNΔ6).

4.6 Recombination sites introduced into the chromosome are functional for recombination.

In order to draw any conclusions from the phenotype of strains containing recombination sites introduced into the bacterial chromosome, it was important to demonstrate that these sites were indeed active for recombination. This could be tested by looking for site-specific recombination between the introduced chromosomal site and a site on a plasmid, causing plasmid integration into the chromosome. By inhibiting plasmid replication, and selecting for a plasmid borne marker, only cells that had a plasmid-copy replicated independently of the plasmid's own origin of replication would be detected. Clearly integration of a plasmid into the host chromosome could result in its replication. Thus, the fraction of cells containing an integrated plasmid would be expected to depend upon recombination between the sites carried on the plasmid and the chromosome. Thus, in an experimental culture in which plasmid replication is inhibited, the detectable levels of plasmid borne antibiotic resistance might be expected to be an indication of plasmid integration (Kuempel *et al.*, 1991).

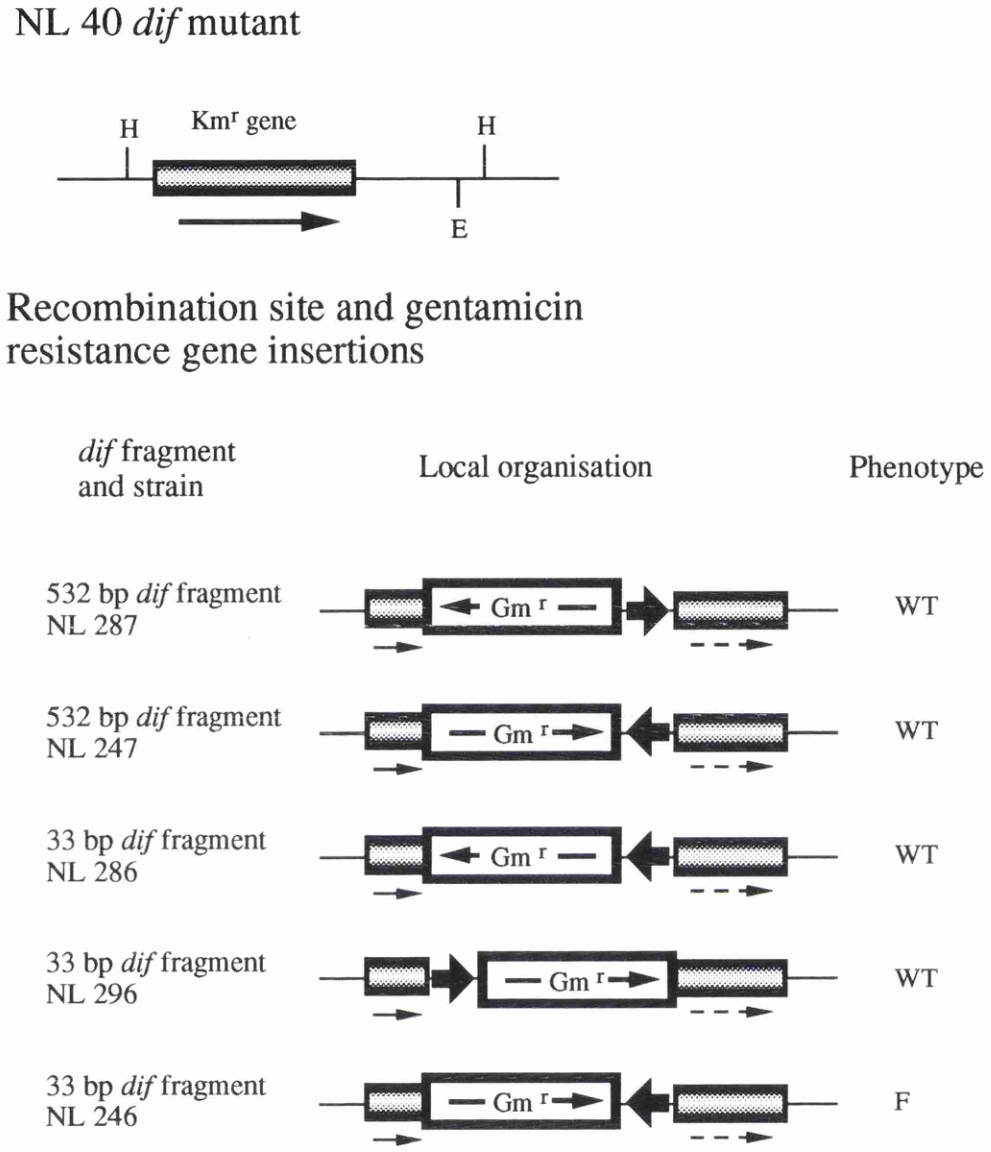


Fig. 4.5. Effects of re-introducing *dif* into the replication terminus. These are diagramatic representations of the chromosomal *dif* regions of various constructed strains. Various *dif* fragments were introduced alongside a gentamicin resistance gene (abreviated to Gm^r) into the terminus of a strain deleted for *dif* (NL40). Construction details, and the phenotype of resultant strains are shown (phenotypes of strains are abreviated, WT - morphologically wild-type and F - filamentous). Strain genotypes are described more fully in the main text. Long thin arrows indicate the direction of transcription of resistance genes. Short fat arrows represent the *dif* fragments inserted, the XerC binding site being closest to the blunt end.

Figure 4.6. Strain verification by Southern hybridisation. Genomic DNA was prepared from each of the strains shown, and digested with the restriction enzyme *Pvu*II.

DS941	wild type
NL40	*difΔ6*
NL41	*difΔ30*
NL244	*difΔ6*::*res*
NL245	*difΔ6*::*cer*
NL246	*difΔ6*::33bp*dif*
NL247	*difΔ6*::532bp*dif*
NL285	*difΔ6*::*cer*
NL287	*difΔ6*::532bp*dif*

Unlabelled lanes contain samples of no relvance to the experiments described.

Samples were separated by 1.0% agarose gel electrophoresis and transferred onto a synthetic membrane by capillary blotting. A radiolabelled *Hin*dIII digest of bacteriophage lambda DNA was included as a marker. Hybridisation was performed using three different DNA fragments, and gave the three separate autoradiographs opposite,

A. An end-labelled 33 bp *dif* core site oligonucleotide.
B. A 243 bp *Eco*RI - *Pvu*II *res* fragment from pMA21, labelled by random primed strand synthesis.
C. A *Bam*HI - *Pst*I *cer* fragment (≈300 bp) from pKS492, labelled by random primed strand synthesis.

Probes were removed with hot alkali, followed by neutralisation before re-probing.

23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
A
B
C
marker
DS941
NL40
NL41
NL244
NL245
NL246
NL247
NL285
NL287
marker

A plasmid with a bacteriophage λdv replication origin cannot replicate if repressed by the lambda *c*I repressor protein (Boyd and Sherratt, 1986). Therefore, transformation of a cell already expressing the *c*I repressor with a λdv based plasmid should only occur at extremely low frequency. However, if there are mechanisms for integration into the bacterial chromosome, it could be replicated without a functional origin. An active site-specific recombination system might be expected to provide this integration, and cause a significant increase in transformation frequency.

An experiment was performed to detect Xer-catalysed recombination between a *dif* containing λdv plasmid and various chromosomal *dif* constructs (DS941, NL40, NLΔIN, NLΔOUT, NL246, NL247, NL286, NL287, and NL296; for construction details see Figs. 3.4 and 4.5). Each strain was made to express λ *c*I by transformation with the *c*I expression plasmid pEA305 (Amann *et al.*, 1983). Competent cells were made of each strain expressing the repressor, as well as plasmid free DS941, and transformed independently with pCB104 (Cm^r λ dv vector, Boyd *et al.*, 1989), pGB310 (pCB104 + 33 bp *dif*, G. Blakely, unpublished work) and pACYC184 (Cm^r, p15A origin, Chang and Cohen, 1978) and plated onto selective media containing ampicillin and chloramphenicol. The differences in transformation efficiency are shown in Table 4.1. This shows extremely few transformants with both lambda-derived plasmids, regardless of the presence of *dif*. Comparisons with the number of transformants recovered without the *c*I expression plasmid, or with pACYC184 show that plasmid replication is being inhibited. However, no increased chloramphenicol resistance by plasmid integration can be detected. If any difference were to exist between the transformation efficiencies of the two λdv plasmids, it could well be masked by the very small numbers involved. The similarity of the data that was produced, however, suggests that *dif*-dependent plasmid integration is not occurring at a level detectable with this assay. One explanation for this absence of detectable recombination could be that all of the components of the recombination system

Strain	Plasmid		
	pCB104	pGB310	pACYC184
DS941	0	1	75
NL40	0	4	64
NLΔIN	0	1	28
NLΔOUT	0	1	54
NL246	5	7	149
NL247	1	1	42
NL286	1	3	25
NL287	0	0	88
NL296	1	1	95
DS941*	368	588	nd

Table 4.1. Will *dif*/Xer recombination allow an inceased transformation efficiency of a strain expressing the λ *c*I repressor with a λdv plasmid? Results shown the number of chloramphenicol resistant colonies recovered in standard transformation. All strains contain the lambda *c*I repressor expression plasmid pEA305, except DS941 denoted *. 'nd' signifies a value not determined; pACYC184 was not used used to transform plasmid free DS941 in this experiment. pCB104 is a λdv plasmid vector, pGB310 is a *dif*$^+$ derivative of this, and pACYC has a p15A origin. Strains used are described fully in the main text and Figs. 3.4 and 4.5.

were present and fully functional, but were not given the opportunity to react due to low transformation efficiency and the single-copy presence of the pGB310 plasmid before this disabled replicon was lost. This problem could be overcome by the use of another means of producing a 'non-replicating circle'.

As an alternative, the plasmid integration method of Kuempel *et al.*, (1991) was used. This method makes use of a temperature-sensitive *repA* gene from the plasmid pSC101, that produces a functional protein at 30°, but not at 42°. Since functional RepA protein is required for plasmid DNA replication , a pSC101 plasmid replicon using this $repA^{ts}$ gene can only initiate replication at permissive temperatures (the replication of pSC101 was recently reviewed by Manen and Caro, 1991). The plasmid pMAK705 (Hamilton *et al.*, 1989) is based upon this temperature-sensitive replicon and carries the chloramphenicol resistance gene from pBR325. Thus, plasmid bearing cells raised to the non-permissive temperature should only grow in the presence of chloramphenicol if a copy of the plasmid is replicated by other means. The most obvious 'other means' would be the integration of a plasmid copy into the host chromosome.

Plasmid integration into the host chromosome would be expected to depend upon significant lengths of homology between plasmid and chromosomal sequences, or the presence of mechanisms for site-specific recombination, or transposition. Kuempel *et al.*, (1991) and our own experiments show that this is the case, as increases in chloramphenicol resistance of up to 5000 fold above a base level were detected. These increases were found to be dependent upon site-specific or homologous recombination, as the absence of either recombination sites, active recombinase enzymes, or a significant length of homology in the system resulted in only a basal number of chloramphenicol resistant colonies.

The fraction (number of Cm^r colonies at 42° / number of Cm^r colonies at 30°) should be representative of the fraction of cells in a particular culture containing a plasmid copy integrated into the chromosome at the time of plating,

or which integrate in the first generations after plating. To help in the performing and interpretation of these experiments, it was important to know how various experimental parameters would affect this perceived integrated fraction. Thus, pilot experiments were performed looking for *dif*-dependent integration of the temperature-sensitive *dif*$^+$ plasmid pLIM701 into the chromosome of DS941. pLIM701 is pMAK705 containing a 231 bp *dif* fragment and was a gift of Peter Kuempel (Kuempel *et al.*, 1991). Significant variability was discovered between different independent calculations of this integrated fraction. 15 independent experiments were carried out investigating the integration of pLIM701 into the chromosome of DS941, with results estimating the fraction of cells containing a plasmid copy integrated into the chromosome ranging from 0.05 to 1.2, giving a mean of 0.46 and a standard deviation of 0.38. Despite this variability, results were not found to be detectably dependent upon the time for which cultures were grown between transformation and plating out, the growth phase of the culture at the time of plating, the original tranformation frequency, nor the time allowed for expression of the Cm^R gene during the tranformation process. These experiments suggest that after transformation, an equilibrium may be quickly achieved between plasmid integration and excision, that is not greatly affected by the growth phase of the bacterial host. Therefore, the calculation of an integrated fraction in a particular experiment may not give an indication simply of the frequency of integration occurring after plasmid replication is inhibited, but also of the position of equilibrium between integrated and free plasmids in the culture at 30° prior to the time of plating. This equilibrium may be complex, depending not only on integration and excision but such factors as the detrimental effects of an active plasmid origin in the bacterial chromosome (Yamaguchi and Tomizawa, 1980). That rates of recombination are in part being analysed is supported by the results with the *cer6* recombination site (Summers, 1989) described in Chapter 5; the integrated fraction is 50 to 70 times higher when recombination should show no selectivity (*argR* and *pepA* strains) compared to

when intramolecular resolution should be favoured (wild-type background). Attempts to assay integration more directly, by analysis of the ratio of Cm^R transformants at 30° and 42° immediately after transformation, were not successful because of the low transformability of the plasmids at all temperatures. The results of integration experiments performed are shown in Table 4.2, also including many results from Chapters 5 and 7.

pMAK705 contains a copy of the Xer recombination site *psi* from pSC101 in its sequence (Kuempel *et al.*, 1991; Cornet *et al.*, 1994). As was found by Kuempel *et al.*, (1991), this site does not appear to interfere with integration experiments, since without other recombination sites introduced into it, pMAK705 gives only basal levels of integrants ($9.0x10^{-4}$ - $4.9x10^{-6}$). Another plasmid, temperature sensitive for replication and containing a *dif* site, very similar to pLIM701 but without *psi* , pFC9 (Cornet *et al.*, 1994) was also used in preliminary integration experiments and gave results consistent with those using pLIM701 (data not shown).

It was important to know that in these plasmid integration experiments, any differences in the number of colonies produced at 30° and 42° were not caused by intrinsic differences in temperature sensitivity, but by differences in plasmid integration. Calculation of the viable cell counts of cultures of different strains at 30° and 42° showed no detectable differences between DS941 (wild-type), DS981 (*xerC*) and NL40 (*dif*) (data not shown).

The dif^+ derivative of pMAK705, pLIM701 (see above) was used to assay the activity of the chromosomal *dif* sites in the strains DS941, NLΔIN, NLΔOUT, NL296, NL246, NL287 and NL247. In all cases, the calculated integrated fractions were not significantly different from each other or DS941, but approximately 5,000 fold higher than experiments with either *dif* or *xer* mutant strains (Table 4.2). This assay shows that the chromosomal *dif* sites in the strains tested are functional for recombination, and does not detect any differences between the strains in the position of the integration/excision equilibrium.

Bacterial strain		Plasmid recombination site			
Name and description	Site	none pMAK705	*dif* pLIM701	*loxP* pN78	*cer6* pN79
DS941, wild-type	*dif*	1.1×10^{-4}	4.6×10^{-1}	3.9×10^{-4}	9.5×10^{-5}
NL40, *dif* deletion	none	5.0×10^{-5}	5.3×10^{-5}	5.3×10^{-4}	4.0×10^{-5}
DS981, *xerC*	*dif*	1.7×10^{-5}	1.3×10^{-5}		
DS9008, *xerD*	*dif*	3.3×10^{-5}	2.3×10^{-4}		
DS902, *recA*	*dif*	3.3×10^{-5}	4.4×10^{-1}		
NL246, *dif* in terminus	33bp *dif*	1.3×10^{-4}	4.0×10^{-1}		
NL247, *dif* in terminus	532bp *dif*	4.9×10^{-6}	3.0×10^{-1}		
NL287, *dif* in terminus	532bp *dif*	1.6×10^{-4}	5.7×10^{-1}		
NL296, *dif* in terminus	33bp *dif*	5.3×10^{-5}	3.7×10^{-1}		
NLΔIN, Km^R by *dif*	*dif*	6.9×10^{-4}	6.3×10^{-1}		
NLΔOUT, Km^R by *dif*	*dif*	7.5×10^{-4}	3.7×10^{-1}		
NL250, *dif* in *lacZ*	532bp *dif*	7.5×10^{-5}	7.1×10^{-3}		
NL350, *dif* near *oriC*	532bp *dif*	1.2×10^{-4}	8.8×10^{-3}		
NL208, *loxP* in terminus	*loxP*	4.0×10^{-4}		6.7×10^{-5}	
NL208 + Cre	*loxP*	9.0×10^{-4}		1.0×10^{-2}	
NL289, *cer6* in terminus	*cer6*	1.5×10^{-5}			4.5×10^{-3}
NL259 (NL289 *pepA*)	*cer6*	1.2×10^{-4}			2.2×10^{-1}
NL279 (NL289 *argR*)	*cer6*	1.3×10^{-5}			3.3×10^{-1}

Table 4.2. Integration of plasmids into the chromosome: calculation of the 'integrated fraction'. These figures are calculated as (number of Cm^R Colonies at 42 / number of Cm^R Colonies at 30°). They represent the fraction of cells in a population containing a plasmid copy integrated into the chromosome, for the strains and plasmids shown. All plasmids used are derived from the Cm^R vector pMAK705, and contain the recombination sites shown. Most results are calculated as the mean fraction from several independent experiments. All strains tested were free of other plasmids, with the exception of 'NL208 + Cre' containing the Cre expression vector pRH200. The *dif* sites in strains DS941, DS981, DS9008 and DS902 are in the natural wild-type context in the terminus region.

4.7 Phenotypic analysis of strains containing *dif* fragments re-introduced into a deletion of the *dif* region

Each strain into which a *dif*$^+$ fragment had been re-introduced into the terminus deletion, and the strains NLΔIN and NLΔOUT, were assayed for the *dif*/*xer* phenotype. Details of strain constructions are shown in Figs. 3.4 and 4.5. Extensive microscopic analysis was carried out on untreated cultures, and after staining of cell nucleoids with DAPI using cells grown with rich or minimal nutrients, on solid media or in liquid culture (Fig. 4.7 and data not shown). In all tests, strains containing the 532 bp *dif* fragment in its natural chromosomal position (NL247 and NL287), two strains similarly containing the 33 bp core site containing fragment (NL286 and NL296), and NLΔOUT (Fig. 3.4) were morphologically indistinguishable from the wild-type strain DS941. However, the strains NL246, containing the 33 bp fragment at the terminus, and NLΔIN (Fig. 3.4) are consistently filamentous, although their phenotypes are generally not as severe as fully mutant strains (*dif*$^-$, *xerC* or *xerD* mutants). Their degree of filamentation is variable and dependant upon the growth of the culture tested. NL246 consistently shows a more severe phenotype than NLΔIN under the same conditions, and both strains show a more severe phenotype when growing exponentially in rich liquid media than when growing on plates.

It was necessary to increase the sensitivity of the microscopic determination of phenotype, to determine phenotypes intermediate between mutant and wild-type. Therefore microscopic preparations were given a subjective "filamentation score" between one and ten (1 = cell length distribution very homogeneous, no filaments, wild-type, 10 = cell length population very variable, many long filaments, mutant). Due to the variability of the phenotype, mutant cultures (*dif*$^-$, *xerC* or *xerD* mutants) were found to have a score of anywhere between seven and ten, wild-type usually between zero and three. To reduce any danger of prejudiced scoring, slide identifying labels were covered

during observation. Representative filamentation scores are presented below in Table 4.3. These values are subjective interpretations of the observed filamentation in several experiments.

Table 4.3

Strain	Subjective filamentation score	
	Exponentially growing cells in rich liquid medium	Cells taken from growth on solid medium
DS941 (wild-type)	1	0
NL40 (*dif*⁻)	9	10
NLΔIN	5	2
NL246	8	5

Thus, the two strains NL246 and NLΔIN contain *dif* core recombination sites positioned in the wild-type location of *dif* in the replication terminus region as judged by Southern blotting experiments. These *dif* sites are functional for plasmid integration and yet both strains display a characteristic *dif* phenotype. What could be the cause of filamentation in these strains, as the related strains, NLΔOUT, NL286 and NL296 are phenotypically wild-type?

One hypothesis to explain the mutant phenotype of these strains suggests that *dif* recombination at the introduced sites is not occurring as efficiently as in wild-type strains. Plasmid integration experiments (in which all strains containing a *dif* site in its natural location including NL246 and NLΔIN gave very similar results) may not be indicative simply of the rate of integrative recombination. However, experiments looking at the excision and loss of integrated non-replicating plasmids over time might be informative about the real rate of excision, as an equilibrium would not be quickly established.

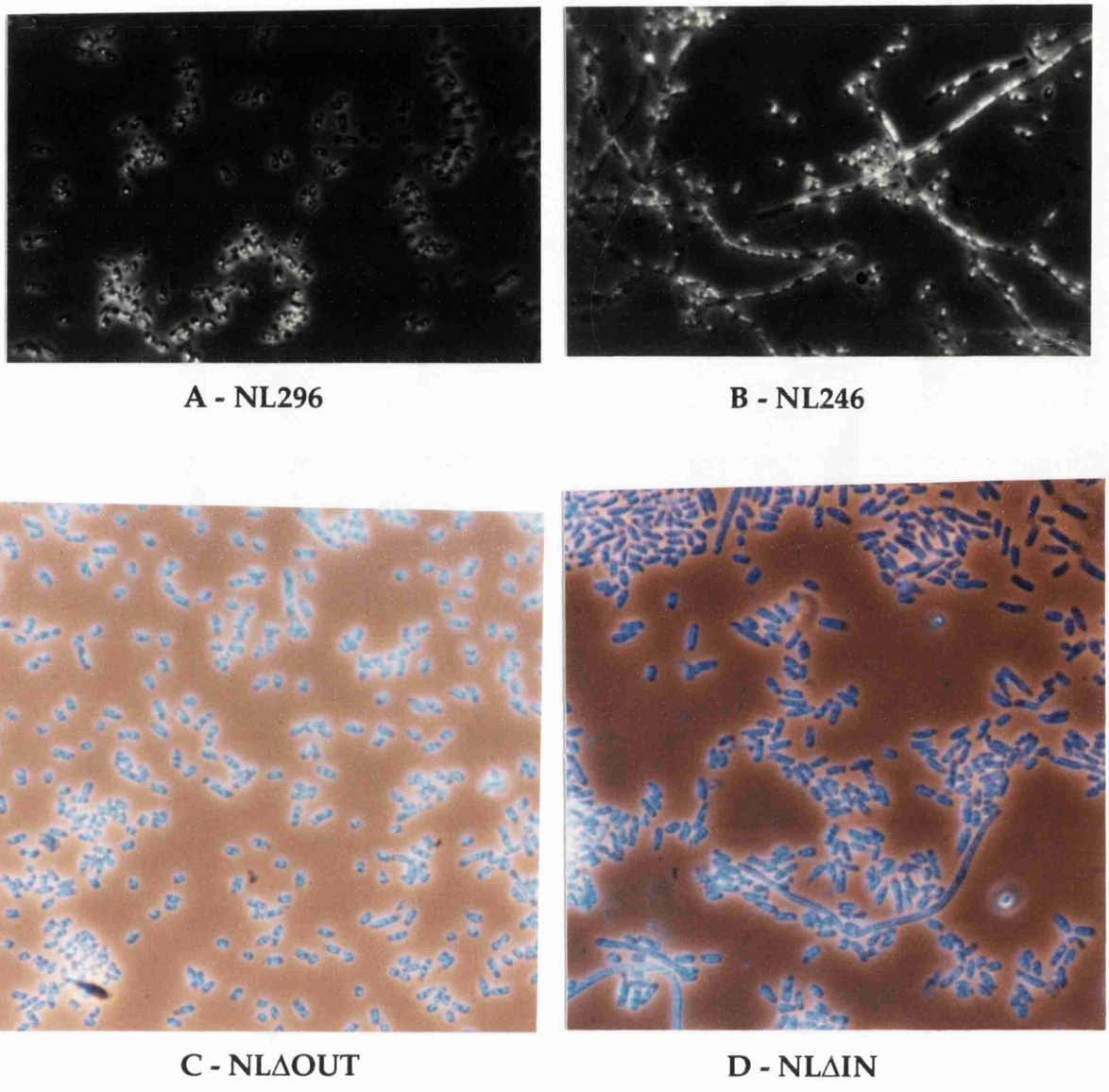


Figure 4.7. **Factors of the local sequence context of *dif* can effect cell and nucleoid morphology.** Exponential phase cultures grown in LB broth at 37° were prepared for photography by condensation and staining of cell nucleoids with chloramphenicol and DAPI respectively (as described in Chapter 3 and Materials and Methods). (**A**) NL296 - 33 bp *dif* re-introduced into chromosomal *dif* deletion. (**B**) NL246 - 33 bp *dif* re-introduced into chromosomal *dif* deletion. (**C**) NLΔOUT - Km^R gene inserted next to *dif*, transcribed away from *dif*. (**D**) NLΔIN - Km^R gene inserted next to *dif*, transcribed towards *dif*. These strains are described more fully in Figs. 3.4 and 4.5. The axes of each photograph represents approximately 70 μm. Condensation of the nucleoids does not interfere significantly with the phenotype demonstrated.

Although preliminary experiments of this kind suggest that at 42°, once selection is removed, NL246 (and indeed NL250, *dif* in *lacZ*, Chapter 7) lose the non-replicating *dif*$^+$ plasmid pLIM701 slower than other strains containing *dif* in the terminus, results were complex and inconsistent, and the experiments time consuming (data not shown).

Although both NL246 and NLΔIN are Xer$^+$ with respect to the plasmid integration assay, it was possible that the phenotypes of these strains might be caused by insufficient expression of, or a mutation in, one of the recombinases XerC or XerD. To test this, expression of both Xer recombinases was provided using the high copy number plasmid pMAY5 (pBAD *XerC*$^+$ *XerD*$^+$, a gift of G. May). Transformation of NL246 with this plasmid had no effect upon the phenotype observed in untreated cultures, with and without the induction of expression using IPTG (data not shown).

Since neither the *dif* loci of these strains, nor the plasmids from which they were derived were verified by nucleotide sequencing, the possibility of novel mutations within these sites cannot be eliminated. If such mutations were present, they would be of great interest, as they would appear to affect cell morphology without affecting measured recombination levels. However, it seems unlikely that the filamentous phenotype of two independently produced strains (NLΔIN, NL246) would be caused by spontaneous mutations in the *dif* site, or in genes affecting *dif* function other than *xerC* or *xerD*, that have no detectable effect upon a plasmid integration assay. Therefore since strains that should contain exactly the same genomic sequences arranged differently display different phenotypes, it seems likely that the local sequence context of the *dif* site is decisive.

One obvious aspect of the local sequence context of the *dif* core sites in these strains is the transcription of the antibiotic resistance genes used in construction. In both NLΔIN and NL246, the chromosomal *dif* core site is positioned directly downstream of an actively transcribed antibiotic resistance

gene (Figs. 3.4 and 4.5). A computer search for possible transcriptional terminator sequences showed that although the gentamicin resistance cassette appears to contain a strong-looking natural terminator after its coding sequence, the kanamycin cassette does not. There were also no terminator sequences detected in any of the *dif* site fragments used. Several factors of the local environment might be effecting *dif*, such as transcription induced supercoiling, local bending or looping of DNA etc. However, at present this is pure speculation. Interestingly, the *dif* core site sequence in pN16, the plasmid used in the construction of NL246, is found to be deleted at relatively high frequency (data not shown). Could this reflect an effect that would cause the lack of *dif* function in the terminus? An investigation of any predicted stable structures formed by the sequences containing and surrounding the *dif* sites of NL246 and NLΔIN (and strains with wild-type morphology) would be useful in addressing these questions.

In order to investigate the possible effects of transcription on *dif* recombination, an attempt was made to perform an *in vivo* recombination assay looking at *dif*-dependent plasmid multimerisation with and without transcription through the *dif* site being provided from an inducible *lac* promoter. The plasmids pMIN33 (Blakely *et al.*, 1991) and pN532 contain *dif* fragments in the polylinker of pUC18 (Fig. 4.3), immediately downstream of the *lac* promoter, that can be artificially induced using IPTG, or repressed with glucose (Yanisch-Perron *et al.*, 1985; Sambrook *et al.*, 1989). Therefore, an *in vivo* plasmid multimerisation assay was performed, growing cells on LB media and ampicillin supplemented with either IPTG (1mM) or glucose (1%). DS902 (*recA*) was transformed with purified monomers of pMIN33 and pN532 and a plasmid DNA preparation made from a mixed pool of transformants of each. This plasmid DNA was then analysed by agarose gel electrophoresis and ethidium bromide staining (Fig. 4.8). This experiment appears to show a subtle increase in multimerisation in cells grown on 1mM IPTG, relative to those grown on 1% glucose. It is possible that

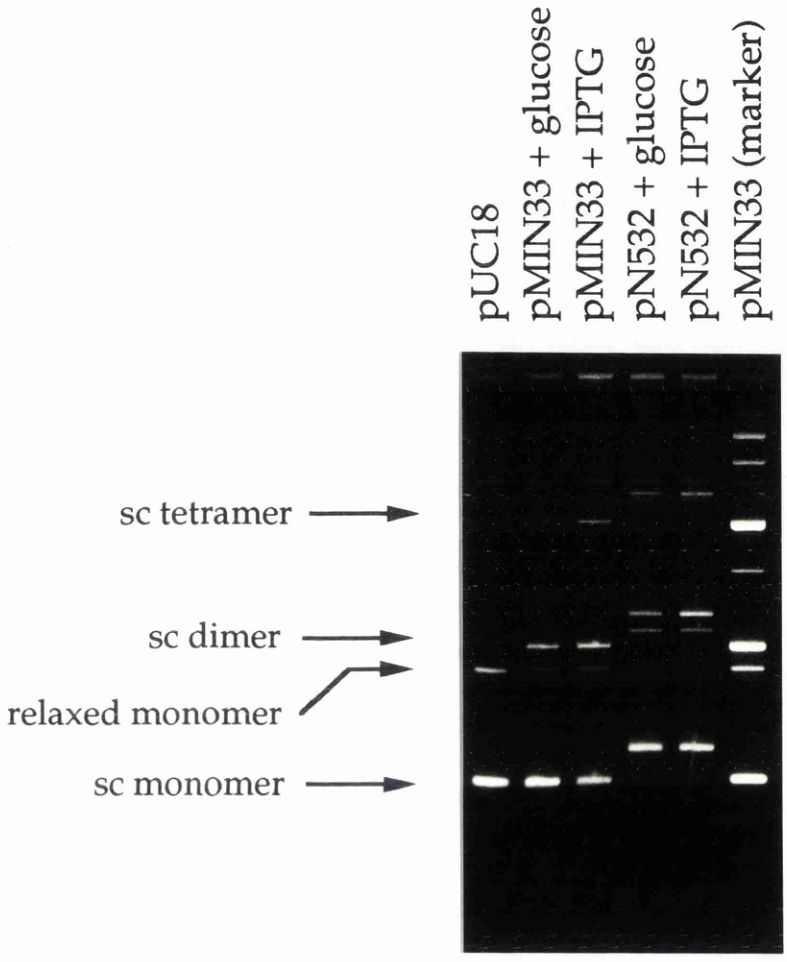


Figure 4.8. **Does induction of transcription across the *dif* site have any effect on *dif* recombination?** In an attempt to investigate the effects of transcription on *dif* recombination, *in vivo* plasmid multimerisation at *dif* was investigated with and without the induction of transcription across the *dif* site. Purified monomers of the *dif*$^+$ plasmids pMIN33 and pN532, both of which contain *dif* in the polylinker of pUC18, were transformed into DS902 (*recA*), and grown with either IPTG (1mM) or glucose (1%). DNA was prepared and analysed by agarose gel electrophoresis, using pUC18 and pMIN33 DNA as markers. Different plasmid forms on the gel, including higher multimeric forms, are labelled, supercoiled being abbreviated to 'sc'. It appears that more multimers are formed in cells grown with IPTG than those grown with glucose.

transcription through the *dif* site in these plasmids, induced by IPTG is causing an increase in plasmid multimerisation. However, this effect is subtle, and could be caused by other factors affected in the experiment, such as the possible drop in plasmid copy number caused by growth on glucose (D. Sherratt, personal communication).

4.8 Discussion

A 33 bp sequence containing the *dif* core site was shown to be able to fully suppress the phenotype caused by a 1500 bp deletion of the chromosomal *dif* locus, when inserted in the replication terminus region in either orientation. Our results show that the chromosomal sequences flanking the *dif* core-site have no detectable effect on *in vivo* plasmid recombination at *dif*, and are not required for normal chromosome segregation. This shows that all of the essential sequences required in *cis* for *dif* function are contained in the 33 bp fragment used. Since all known recombination systems that display recombination selectivity have sequences flanking a core recombination site that are required for, or profoundly effect, site-specific recombination in plasmid substrates (e.g.. *cer*, λ *att*, *res*, *gix*), this is more evidence that Xer-catalysed recombination at *dif* does not display selectivity in the manner of other well-studied recombination systems (e.g.. Tn*3* *res* or ColE1 *cer*). This requires that if the function of *dif* is to convert chromosome dimers to monomers, this selectivity must be enforced by an entirely novel mechanism not requiring sequences immediately flanking *dif*, and not evident in plasmid recombination experiments. If this is true, chromosome monomerisation must be directed by other means.

These results also show that the chromosomal orientation of *dif* is unimportant for its function. This would argue that *dif* could not be part of a site-specific inversion system. An inversion system would also require two

recombination sites in inverted repeat, and would therefore presumably need another copy of *dif*, or a very similar sequence in the chromosome. Experiments hybridising radio-labelled *dif* core site oligonucleotides to genomic DNA have failed to detect any other strongly hybridising sequences. This result is also evidence against any other hypothesis for *dif* function that requires the existence of another site similar to *dif*.

Chapter 5

Can other recombination systems suppress the *dif* phenotype?

Introduction - Can other recombination systems suppress the *dif* phenotype?

The hypothesis has been proposed that *dif* exists to resolve chromosome dimers to monomers by site-specific recombination, prior to cell division. If this is true, it might be expected that other site-specific recombination systems with similar properties could carry out the same biological function. The suppression of the *dif* mutant phenotype by other recombination systems would be good evidence that the function of *dif* really is to provide recombination, and might also be informative about the characteristics of the recombination required. Therefore *dif* was replaced by the introduction of other recombination sites into the replication terminus, and the expression of any required recombinases was provided by appropriate plasmids. A range of well characterised recombination systems with different recombinational properties were used. Both *cer*/Xer of the plasmid ColE1 (Summers and Sherratt 1984; Sherratt *et al.*, 1995), and *res*/resolvase of transposon Tn*3* (Stark *et al.*, 1989b) were used, as they display strict resolution selectivity. On the other hand, *loxP*/Cre of bacteriophage P1 was used as an example of an unconstrained recombination system. The artificial ColE1 *cer*/CloDF13 *parB* hybrid site, *cer6* was used, as it is also a substrate for the Xer proteins, and displays predictably different recombinational properties; recombination shows some resolution selectivity only in the presence of accessory proteins and sequences, but occurs in an unconstrained manner in their absence (Summers, 1989). Recombination sites were introduced into the chromosome using the same strategy as was used for the re-introduction of *dif* fragments in Chapter 4 (Figs. 3.1 and 4.4). In an attempt to avoid the local sequence context of recombination sites interfering with their ability to functionally replace *dif*, certain arrangements of introduced sequences in which

dif core sites seemed unable to allow normal chromosome segregation were avoided (Chapter 4).

5.1 Transposon Tn*3* *res*/resolvase

The transposon Tn3 contains a site-specific resolution system consisting of a 114 bp recombination site, *res*, and a resolvase enzyme, encoded by the *tnpR* gene, a member of the resolvase/invertase family of site-specific recombinases (reviewed by Grindley, 1994, Sherratt, 1989, and Stark *et al.*, 1989b). Results of experiments using the closely related *res*/resolvase system from the transposon γδ are widely accepted to be applicable to both systems (Grindley, 1994); some are considered here. These systems function to resolve co-integrate molecules produced during replicative transposition, and therefore display strong resolution selectivity both *in vitro* and *in vivo*, efficient recombination being catalysed only between sites in direct repeat in the same supercoiled molecule (Reed, 1981; Stark *et al.*, 1989b; Bliska *et al.*, 1991). If a single *res* site were introduced into the chromosomal replication terminus in place of *dif*, the two copies in a chromosome dimer would indeed be in direct repeat in the same supercoiled molecule. However, the extreme distance between the sites (4.7 Mb of DNA) might be expected to interfere with the resolution selectivity mechanism, as discussed below.

The plasmid pMA1441 (pUC18 + *res*, a gift of Martin Boocock) was used as a source of *res* site DNA. A *res*$^+$ *Kpn*I - *Xba*I fragment (≈200 bp) from this plasmid was cloned next to the gentamicin resistance gene of pN10 to give plasmid pN14. This Gm^R*res*$^+$ fragment was then cloned into the deletion of the chromosomal *dif* site in pNΔ6 (Figs. 3.5 and 4.4) giving pN24 (*difΔ6*::*res*Gm^R). This mutation

was introduced into the chromosome by linear transformation (Fig. 3.1 and Chapter 3), then transduced out of the *recBC sbcBC* background to give NL224 (DS941 *difΔ6*::*res*Gm^r) and NL244 (NL40 *difΔ6*::*res*Gm^r). Details are shown in Fig. 5.1. That a *res* site had indeed been introduced into the *dif* locus was verified by Southern hybridisation (Fig. 4.6). A radio-labelled *res*$^+$ fragment from pMA21 (Bednarz *et al.*, 1990) showed hybridisation to an NL244 genomic *Pvu*II fragment of approximately 10.5 kb. A band of this size also showed hybridisation in an earlier experiment with a labelled fragment of chromosomal DNA flanking the *dif* locus (taken from pN1), and is the size predicted by the physical map of the *E.coli* chromosome (Kohara *et al.*, 1987). Plasmid pMA21 was used as a fresh source of *res* DNA for labelling, not pMA1441, the origin of the site introduced into the chromosome.

Recombination at *res* is catalysed by the resolvase enzyme of Tn*3*, encoded by the *tnpR* gene. The *E.coli* strains used (AB1157 derivatives) do not include a Tn*3* element (or one closely related to Tn*3*). Therefore, for any recombination to occur at the site in NL244, resolvase expression must be provided. The plasmid pPAK316 was used as a source of resolvase. This is simply pACYC184 containing a deleted transposition defective Tn*3* element (Kitts *et al.*, 1983). Resolvase is expressed at a low level from its natural promoter, and auto-regulated by its binding to the natural *res* site of Tn*3* which overlaps this promoter (Reed *et al.*, 1982). The successful expression of Tn*3* resolvase from pPAK316 in NL244 was verified by a reporter plasmid resolution assay, using the two *res* plasmid pMA21 (Bednarz *et al.*, 1990). This plasmid, based upon pBR322, contains two Tn*3* *res* sites in direct repeat. Recombination between the two copies of *res* can be detected through deletion of the intervening DNA. When transformed into NL244, recombination was detected in the presence of pPAK316, but not in its absence (Fig. 5.2). This shows that the expression of resolvase in this strain is

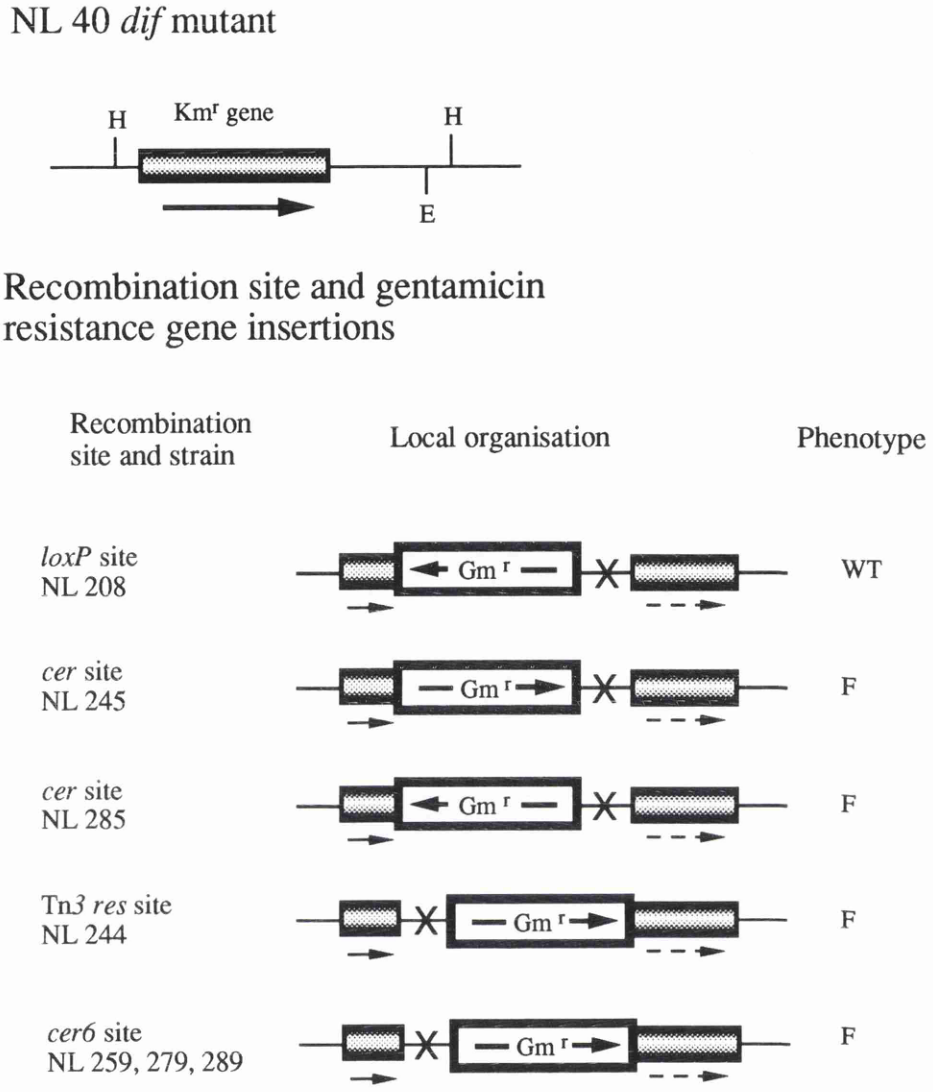


Figure 5.1. Effects of re-introducing recombination sites into the replication terminus. These are diagramatic representations of the chromosomal *dif* regions of various constructed strains. Various recombination sites were introduced alongside a gentamicin resistance gene (abreviated to Gm^r) into the terminus of a strain deleted for *dif* (NL40). Any required recombinase enzymes were expressed from plasmids, as described in the main text. Construction details, and the phenotype of resultant strains are shown (phenotypes of strains are abreviated, WT - morphologically wild-type and F - filamentous). Strain genotypes are described more fully in the main text. Long thin arrows indicate the direction of transcription of resistance genes. Crosses represent the recombination sites inserted.

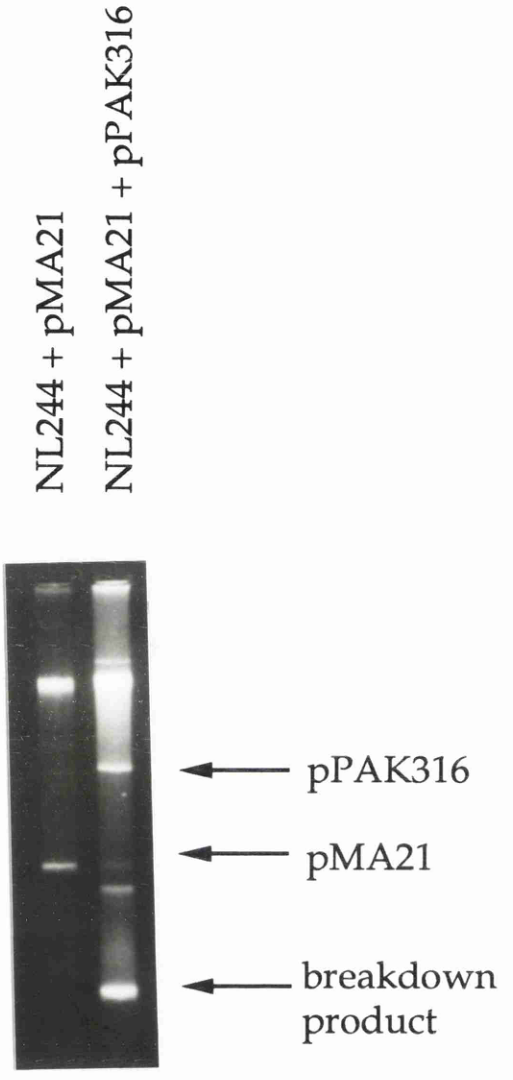


Figure 5.2. Expression of Tn*3* resolvase in NL244. The strain NL244 (Tn*3* *res* in place of *dif*) was transformed with pMA21, and with pMA21 and pPAK316. Plasmid DNA was prepared and analysed by agarose gel electrophoresis. pMA21 is a reporter plasmid containing two Tn*3* *res* sites in direct repeat (Bednarz *et al.*, 1990). pPAK316 (Kitts *et al.*, 1983) expresses Tn*3* resolvase. pMA21 and pPAK316 are 4.9 and 8.6 kb in size respectively. Breakdown of pMA21 is only evident in the presence of the resolvase expression plasmid pPAK316.

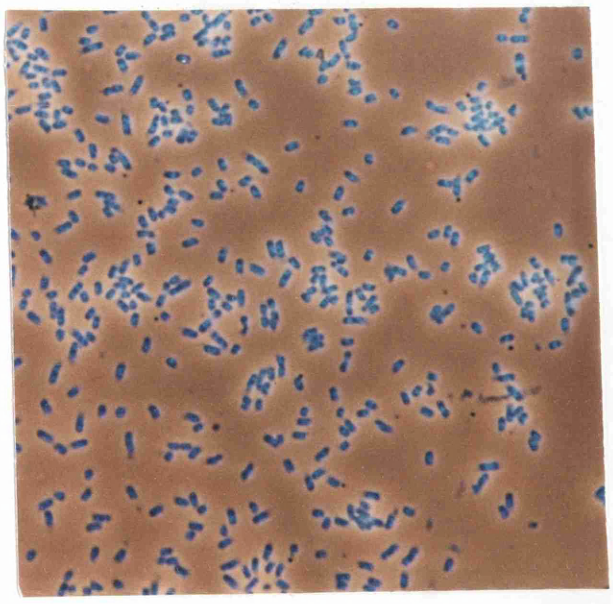
sufficient for full resolution of pMA21. Since this plasmid is present in high copy number, this seems to suggest that recombination at the single chromosomal site in NL244 would not be limited by resolvase availability. The recombination selectivity shown by the Tn3 *res*/resolvase system means that the activity of the *res* site introduced into the chromosome in NL244 cannot be demonstrated by the previously described plasmid integration method.

Cultures of NL244 transformed with the resolvase expression plasmid pPAK316 were grown in liquid culture or on solid media, with antibiotic at 37° and observed under the microscope, both untreated, and after cell nucleoids had been stained using DAPI (Chapter 3 and Materials and Methods). Cultures were always found to display the mutant phenotype characteristic of *dif*/Xer mutant cultures (Fig. 5.3).

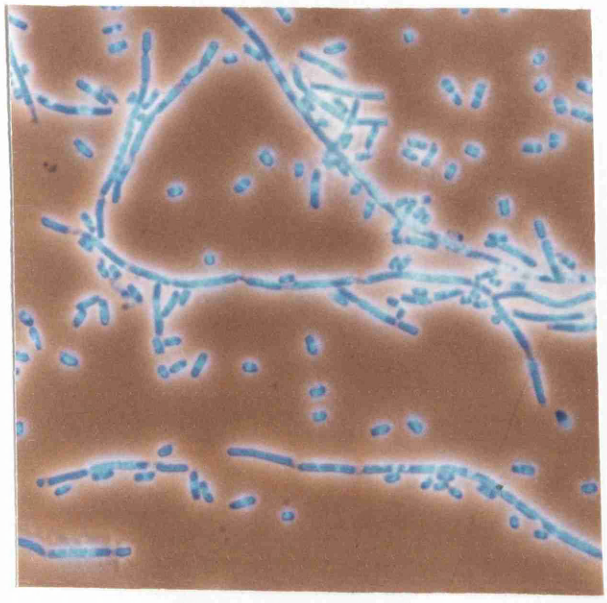
The *dif*/Xer phenotype caused by deletion of the *dif* site is not suppressed by the introduction of a Tn3 *res* site into the replication terminus, and expression of resolvase. This suggests that the Tn3 *res*/resolvase system is unable to functionally replace the *dif*/Xer system in the *E.coli* chromosome.

5.2 Plasmid ColE1 *cer*

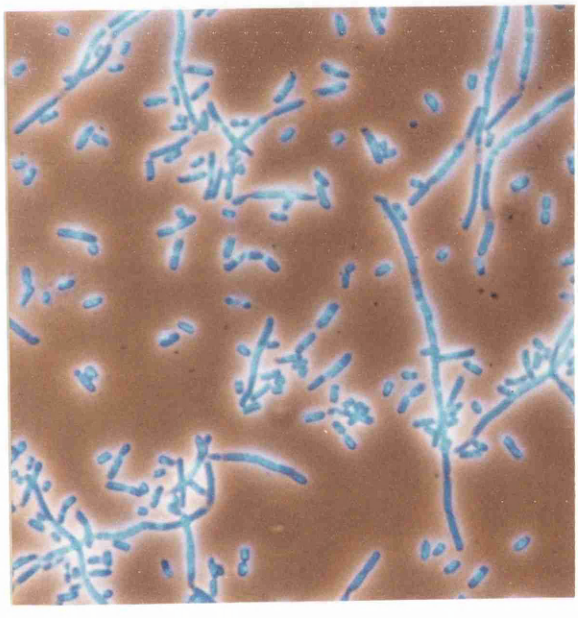
Recombination at the *cer* site of the natural high copy number plasmid ColE1 is catalysed by the chromosomally encoded recombinases XerC and XerD of *E.coli*, and converts plasmid dimers to monomers (Summers and Sherratt, 1984). *cer* recombination has been extensively studied *in vivo*, although a full recombination reaction has not been reconstituted *in vitro*. As with Tn3 *res*, *cer* recombination uses strict resolution selectivity to fulfil its biological role *in vivo*, recombining sites separated by only the length of the ColE1 plasmid (6.65 kb). In



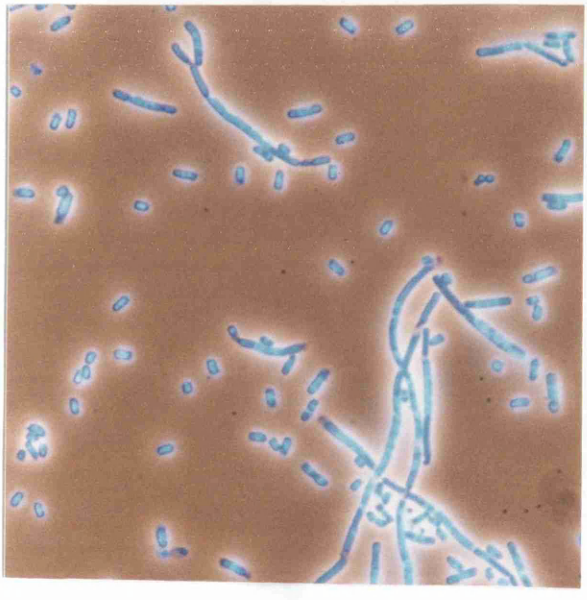
A - DS941



B - NL244



C - NL285



D - NL259

Figure 5.3. Microscopic analysis of cell and nucleoid morphology. Exponential phase cultures grown in LB broth at 37° were prepared for photography by condensation and staining of cell nucleoids with chloramphenicol and DAPI respectively (as described in Chapter 3 and Materials and Methods). (**A**) DS941 - wild-type. (**B**) NL244 - Tn*3* *res* site in place of *dif* with pPAK316 resolvase expression plasmid. (**C**) NL285 - ColE1 *cer* in place of *dif* (**D**) NL259 - full *cer6* site in place of *dif, pepA* background. The axes of each photograph represents approximately 70 μm. Condensation of the nucleoids does not interfere significantly with the phenotype demonstrated.

order to determine whether *cer* can functionally replace *dif* in the chromosome, it was necessary to introduce a *cer* site into the replication terminus in place of *dif*. A similar strategy was used as for *res* and *dif* fragments.

pKS492 (Stirling *et al.*, 1988b) was used as a source of *cer* DNA, containing a 280 bp *Hpa*II-*Taq*I fragment from ColE1 including the full length *cer* site sequence (Summers and Sherratt, 1984, 1988). The results from Chapter 4 suggest that the orientation of antibiotic resistance genes used in construction may be able to have some effect on phenotypic suppression by introduced recombination sites. Therefore, constructs were designed with *cer* at each end of the Gm^R gene used. A *Bam*HI - *Pst*I fragment of pKS492 (≈300 bp) was incorporated into pN15 and pN55, to give two easily movable Gm^r cer^+ fragments. As with earlier experiments, these Gm^r cer^+ fragments were cloned into pNΔ6 to give constructs *difΔ6*::*cer* Gm^r1 and *difΔ6*::*cer* Gm^r2 respectively (Chapter 4 and Fig. 4.4). These constructs were then introduced into the chromosome by linear transformation and transduced into DS941 and NL40 (DS941 *difΔ6*) to give NL225 (DS941 *difΔ6*::*cer*Gm^R1), NL245 (NL40 *difΔ6*::*cer*Gm^R1), NL265 (DS941 *difΔ6*::*cer*Gm^R2), and NL285 (NL40 *difΔ6*::*cer*Gm^R2). Details of constructs are contained in Fig. 5.1 and Tables 2.1 and 2.2.

These four strains, NL225, 245, 265 and 285 are all dif^- and contain *cer* in the replication terminus in place of *dif*. NL245 and NL285 were verified by Southern blotting (Fig. 4.6). A radio-labelled cer^+ fragment from pKS492 showed hybridisation to genomic *Pvu*II fragments of approximately 10.5 kb from NL245 and NL285. Bands of this size also show hybridisation in a previous experiment with a labelled fragment of chromosomal DNA flanking the *dif* locus (taken from pN1), and are the size predicted by the physical map of the *E.coli* chromosome (Kohara *et al.*, 1987).

The activity of the chromosomally encoded recombinases XerC and XerD in these strains was verified by *in vivo* recombination assays. The reporter plasmid pKS455 is based upon pUC9, and contains two directly repeated *cer* sites flanking a chloramphenicol resistance gene (Fig. 4.1; Stirling *et al.*, 1988a). Introduction into an *xer*$^+$ strain results in recombination between the sites and deletion of the resistance gene, to give pKS456. pKS455 was transformed into DS984 (*xerC*), NL245 and NL285 and plasmid DNA analysed by gel electrophoresis (Fig. 5.4). In NL245 and 285, only pKS456 could be detected, whereas in DS984, only pKS455 could be seen. The selectivity of *cer* recombination means that the recombinational activity of the *cer* site introduced into the chromosome cannot be verified by the plasmid integration method used previously, as recombination will not be catalysed between sites on different molecules.

Cultures of NL245 and NL285 were studied to determine whether they display the *dif/xer* phenotype. Cultures were grown in liquid culture with shaking, and on solid medium, at 37° and microscopically observed both untreated, and after staining of nucleoids with DAPI. Both strains with *cer* in place of *dif* display the *dif/xer* phenotype (Fig. 5.3). This strongly suggests that *cer* is unable to functionally replace *dif* in the *E.coli* chromosome.

5.3 The ColE1 *cer*/ClodF13 *parB* hybrid site - *cer6*

Summers (1989) described a hybrid Xer recombination site, the type II hybrid, produced by low frequency recombination between the *cer* site of plasmid ColE1 and the related site, *parB* of ClodF13 (Hakkaart *et al.*, 1984). As the XerC and XerD binding sequences of this site are identical to those of *cer*, but are

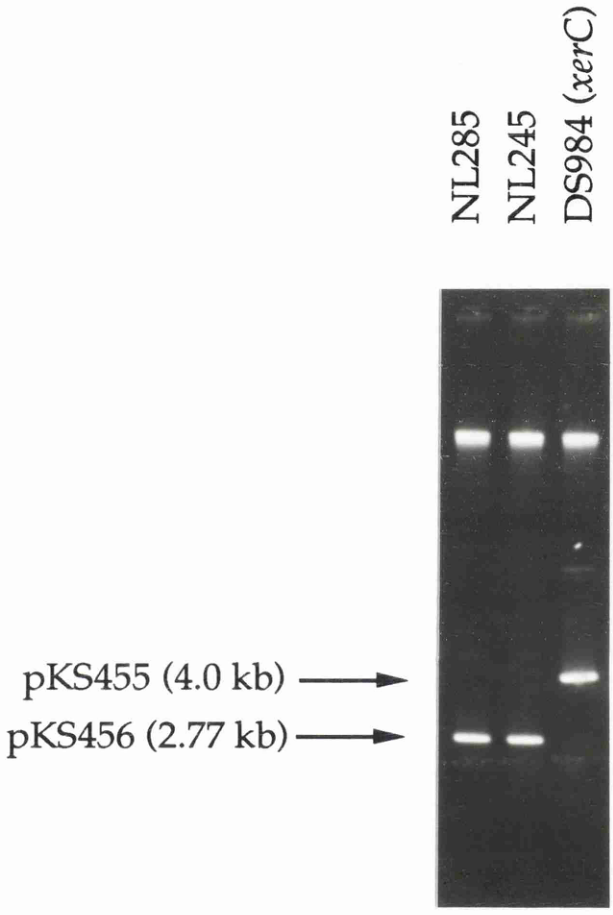


Figure 5.4. Expression of the Xer proteins in NL245 and NL285. The two *cer* site reporter plasmid pKS455 was used to assay the activity of the chromosomally encoded Xer proteins in NL245 and NL285 (two strains with *cer* in place of *dif*). The strains DS984 (*xerC*), NL245 and NL285 were transformed with pKS455. Plasmid DNA was prepared and analysed by agarose gel electrophoresis. pKS455 is a reporter plasmid containing two *cer* sites in direct repeat (Fig. 4.1). Xer-dependant breakdown of pKS455 to give pKS456 is evident in both NL245 and NL285, showing that the Xer proteins are actively expressed in these strains.

separated by 6 bp of unrelated DNA rather than the normal 8 bp of *cer*, this site is here called *cer6*. Recombination at this site displays some resolution selectivity in the presence of the *cer* accessory proteins, ArgR and PepA. However, in the absence of either ArgR, PepA, or the accessory DNA sequences flanking the core recombination site, this selectivity is abolished, and recombination occurs both inter- and intra-molecularly.

A full length *cer6* site was used to replace *dif* in the *E.coli* chromosome, as it might be expected that this site could suppress a *dif* deletion phenotype in the absence of accessory proteins, but not in a wild-type background. The original plasmid of Summers, (1989), 'pUC8 + type II hybrid' was used as a source of DNA. The substrate activity of the plasmid isolate used was verified by observing Xer and *cer6* dependent multimerisation of plasmids (data not shown). A *cer6*$^+$ *Nde*I - *Hin*dIII fragment of approximately 450 bp was used, with non-essential sequences flanking the recombination site on both sides. This site was cloned next to the Gm^R gene of pN10, giving pN39, for easy manipulation of a *cer6*$^+$ Gm^R fragment, that was cloned into the Km^R gene of pNΔ6 to give pN69 (*difΔ6*::*cer6*Gm^R). This mutation was introduced into the replication terminus in place of *dif* as described for *res* and *cer*. Construction details are contained in Fig. 5.1 and Tables 2.1 and 2.2. This *cer6* terminus construct was transduced into four different strains, DS941, NL40 (*dif*), DS956 (*argR*) and DS957 (*pepA*), to give NL269, NL289, NL279 and NL259 respectively. These constructs were verified by Southern hybridisation using labelled *cer6* and *dif* site probes (data not shown). Recombination at *cer6* is mediated by the chromosomally encoded proteins XerC, XerD, ArgR and PepA. The activity of these proteins in each strain was determined using the two *cer* reporter plasmid pKS455 (Fig. 4.1; Stirling *et al.*, 1988a) and the plasmid integration assay used previously (Chapter 4). NL289, NL259, NL279 and the filamentous Xer$^+$ strain NL250 were transformed with

pKS455 and DNA was prepared after two periods of overnight growth. Full recombination between the two copies of *cer* to give pKS456 occurred in the Xer^+ strains NL250 and NL289, but not in NL279 (*argR*) or NL259 (*pepA*) (Fig. 5.5).

The plasmid integration assay used previously (Kuempel *et al.*, 1991; Chapter 4) was used to demonstrate the recombinational activity of the *cer6* site introduced into the chromosome. This experiment would also demonstrate the activity of the recombinases XerC and XerD in the strains unable to support *cer* recombination in pKS455, NL279 and NL259. For this assay, a *cer6* derivative of the replication temperature-sensitive Cm^R plasmid pMAK705 was produced. As above, a $cer6^+$ fragment of approximately 450 bp was used, and cloned into the polylinker of pMAK705, to give pN79. Experiments were conducted as described previously (Chapter 4). The relative numbers of chloramphenicol resistant colonies at 30° and 42° in these experiments depends upon recombination between copies of *cer6* in the chromosome and in pN79, as shown by the results in the following table, an extract from Table 4.2. The *cer6* site in the terminus region in place of *dif* is therefore functional for recombination. The results of these experiments will discussed below in Table 5.1.

Table 5.1

Bacterial strain		Plasmid recombination site	
Name and description	Site	none pMAK705	*cer6* pN79
DS941 (wild-type)	*dif*	$1.1x10^{-4}$	$9.5x10^{-5}$
NL40 (*dif* deletion)	none	$5.0x10^{-5}$	$4.0x10^{-5}$
NL289 (*cer6* in terminus)	*cer6*	$1.5x10^{-5}$	$4.5x10^{-3}$
NL259 (NL289 *pepA*)	*cer6*	$1.2x10^{-4}$	$2.2x10^{-1}$
NL279 (NL289 *argR*)	*cer6*	$1.3x10^{-5}$	$3.3x10^{-1}$

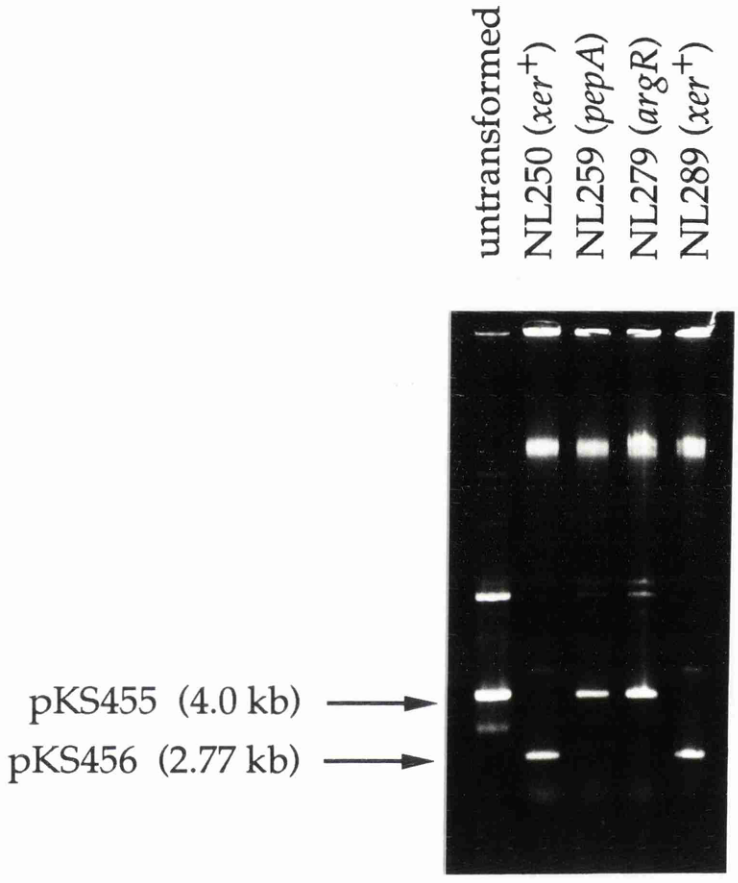


Figure 5.5. The activity of the Xer proteins in NL259, NL279 and NL289. The two *cer* site reporter plasmid pKS455 was used to assay the activity of the chromosomally encoded Xer proteins in NL259 (*pepA*), NL279 (*argR*) and NL289 (*xer*$^+$). All three strains contain a *cer6* site in place of *dif* in the chromosome. The strains NL250 (filamentous *xer*$^+$), NL259, NL279 and NL289 were transformed with pKS455. Plasmid DNA was prepared and analysed by agarose gel electrophoresis. pKS455 is a reporter plasmid containing two *cer* sites in direct repeat (Fig. 4.1). Xer-dependant breakdown of pKS455 to give pKS456 is evident in NL250 and NL289. This data is consistent with the genotypes of these strains.

Cultures of NL289, NL269, NL279 and NL259 were observed untreated and after staining of nucleoids with DAPI, to determine whether they display the cell morphology phenotype of *dif*/Xer mutants (Fig. 5.3 and data not shown). In all experiments, all four strains were not significantly different from the standard *dif* mutant NL40. Therefore in these experiments, in neither wild-type, *argR*, nor *pepA* genetic backgrounds is recombination at *cer6* able to functionally replace *dif* in the replication terminus region. Possible explanations for this will be discussed below.

5.4 Bacteriophage P1 *loxP*/Cre

The *loxP*/Cre recombination system of bacteriophage P1 is an example of a simple, non-selective recombination system (Hoess and Abremski, 1990), although its biological role is unclear, despite being implicated in cyclisation of 'phage DNA, integration into the host chromosome, plasmid dimer resolution and plasmid amplification (Austin *et al.*, 1981; Yarmolinsky and Sternberg, 1988; Adams *et al.*, 1992). It has been extensively studied *in vitro* and been shown to catalyse fusion, resolution or inversion reactions between sites in supercoiled, relaxed, or linear DNA molecules. As with XerC and XerD, Cre is a member of the integrase family of site-specific recombinases. More recent results have called into question whether Cre recombination is as unconstrained in plasmid substrates *in vivo* as seen *in vitro* (Adams *et al.*, 1992).

A *loxP* site was introduced into the terminus region in place of *dif* by similar methods used for previous sites. Sternberg *et al.*, (1983) defined the 34 bp of *loxP* sequences required for substrate activity in recombination, although there is some evidence that sequences flanking this site in 'phage P1 have some effect

on recombination *in vivo* (R. Hoess and D. Sherratt, personal communication). Two complementary oligonucleotides containing this 34 bp *loxP* sequence, and incorporating a convenient diagnostic *Xho*I restriction enzyme site were kindly supplied by Marshall Stark (O1 and O2, Table 2.3). These oligonucleotides were annealed and ligated into the polylinker of pMTL23 to give pN40. Cloning of a gentamicin resistance gene next to the site created a *loxP* Gm^R fragment (in pN38) that could be introduced into terminus region DNA in place of *dif* as before. This *loxP*/marker fragment was cloned into the coding sequence of the Km^R gene of the deleted *dif* locus plasmid pNΔ6 to give pN68, *difΔ6*::*loxP*Gm^R. An *in vitro* recombination assay was used to demonstrate the activity of the *loxP* site in this plasmid as a substrate for Cre-mediated recombination. Plasmid DNA of pN68, and pN69, a similar plasmid containing a *cer6* site instead of *loxP*, was incubated with and without purified Cre protein, as described in Materials and Methods, and visualised by agarose gel electrophoresis (Fig. 5.6). The results clearly show Cre-dependent recombination and nicking of pN68, with pN69 being unaffected.

Mutagenesis using a temperature sensitive plasmid replicon. Previous mutations had been introduced into the bacterial chromosome by homologous replacement of the wild-type *dif* locus in strains deficient for exonuclease V, followed by generalised transduction using bacteriophage P1 into other genetic backgrounds. The *loxP*/Cre recombination system is native to bacteriophage P1. 'Phage P1 expresses Cre, and contains a *loxP* site within its genome. It would therefore be possible that infection by 'phage P1 of a strain containing a *loxP* site in the terminus region would cause unwanted recombination between copies of the site, with unknown consequences. Therefore it was decided to introduce this mutation into the bacterial chromosome by other means.

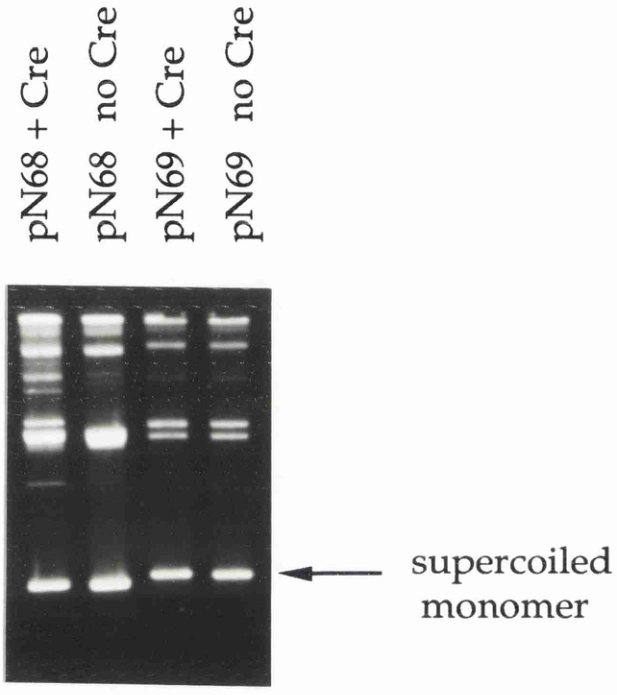


Figure 5.6. The effect of Cre protein on pN68 and pN69 *in vitro*. An *in vitro* recombination assay was performed using purified Cre protein and the two substrates pN68 (*loxP*$^+$) and pN69 (no *loxP* site). Each plasmid DNA was incubated with and without Cre protein at 37° for an hour, then analysed by gel electrophoresis. Incubation with Cre appeared to have no effect upon pN69, but with pN68 substantial multimerisation, nicking and linearisation of the plasmid seems to have occurred. This is strong evidence that the *loxP* site in pN68 is an active substrate for Cre-mediated recombination.

Hamilton *et al.*, (1989) describe the use of plasmids derived from pSC101, carrying a chloramphenicol resistance gene, which are temperature sensitive for replication and can be used to select for plasmid integration into the chromosome. One such plasmid, pMAK705 has been used here and by Kuempel *et al.*, (1991) to test the functioning of site-specific recombination sites introduced into the chromosome. However, the plasmids were originally designed for chromosomal mutagenesis by selecting for homologous replacement. A plasmid borne mutation can be forced to integrate into a wild-type chromosomal homologue by incubation at the non-permissive temperature and selection with chloramphenicol. This produces a co-integrate chromosome with two copies of the locus to be mutated. In turn, excision can then be selected for by returning the cells to the permissive temperature, as an active replication origin integrated into the bacterial chromosome has a significant detrimental effect on cell growth (Yamaguchi and Tomizawa, 1980).

This method has the advantage over the 'linear transformation' method used elsewhere in this work, as it will function in most commonly used laboratory bacterial strains, and therefore does not require subsequent transduction of mutations. However, mutations must first be introduced into the temperature sensitive vectors, and also the subsequent selection for the correct excision of the wild-type sequence may be a lengthy process. Since the construct to be introduced to the chromosome contained a Gm^R gene within the DNA to be introduced, it was possible to slightly adapt the method of Hamilton *et al.*, (1989). By repeated cycling of cells between permissive and non-permissive temperatures whilst selecting for gentamicin, it was possible to select for repeated excision of any plasmid in the chromosome, and integration of any gentamicin resistant plasmid. Therefore, each cycle would increase the probability that the

original wild-type sequences had been lost, along with the temperature sensitive plasmid.

In order to use this method, the *difΔ6::loxP*Gm^R construct, a *loxP* site and Gm^R gene flanked on both sides by chromosomal sequences flanking *dif*, was cloned into the polylinker of pMAK705, to give pN58. This mutation was then successfully introduced into the chromosome of the *dif* mutant NL40 to give strain NL208 (Fig. 5.1). Attempts were also made for its introduction into DS941 (*dif*$^+$), however, these proved unsuccessful; of 200 Gm^R colonies analysed after temperature cycling, none were found to be chloramphenicol sensitive (whereas with NL40, 15 of 40 colonies analysed were Gm^R Cm^S Km^S). The construction of NL208 (and NL202 and NL203, Chapter 7) was verified by Southern hybridisation. Genomic DNA was digested with *Pvu*II, separated by agarose gel electrophoresis, transferred to a synthetic membrane and sequentially hybridised with two radio-labelled probes, firstly a *loxP* sequence oligonucleotide and secondly a 532 bp *dif*$^+$ fragment. The *loxP* probe was found to hybridise to a band of the size predicted by the physical map of the chromosome with strain NL208, and also with NL202 and NL203 (Fig. 5.7 and data not shown).

Suppression of the *dif* deletion phenotype by the *loxP*/Cre system. The Cre recombinase was expressed from the plasmid pRH200, a pUC based plasmid containing the Cre gene downstream of its natural promoter (Mack *et al.*, 1992). The previously used plasmid integration method (Kuempel *et al.*, 1991; Chapter 4) was used to demonstrate the expression of active Cre from pRH200, and the recombinational activity of the *loxP* site in the chromosome of NL208. Therefore, a *loxP* derivative of the temperature-sensitive plasmid pMAK705 was produced by cloning a *loxP*$^+$ fragment of approximately 60 bp into the polylinker, to give

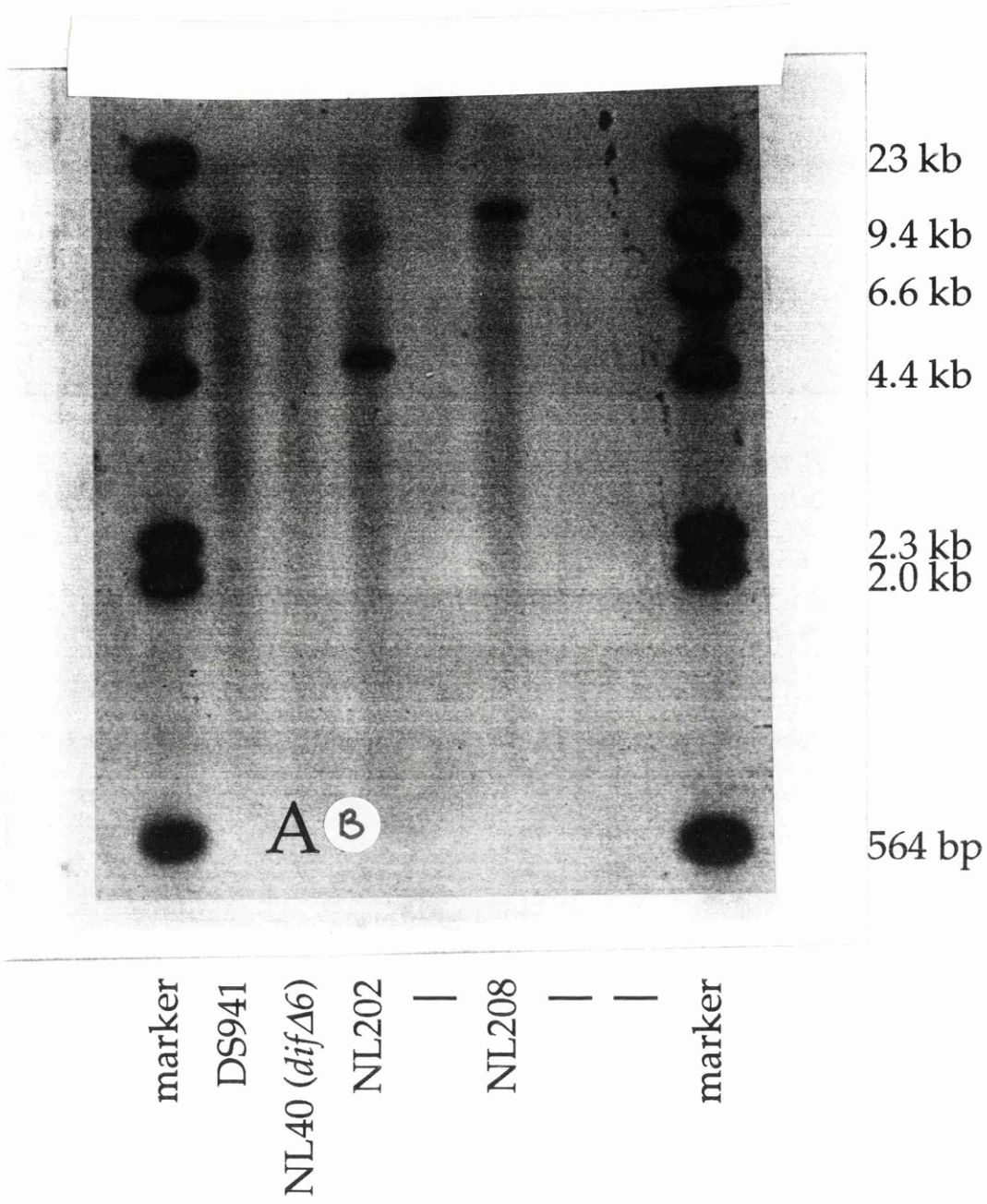
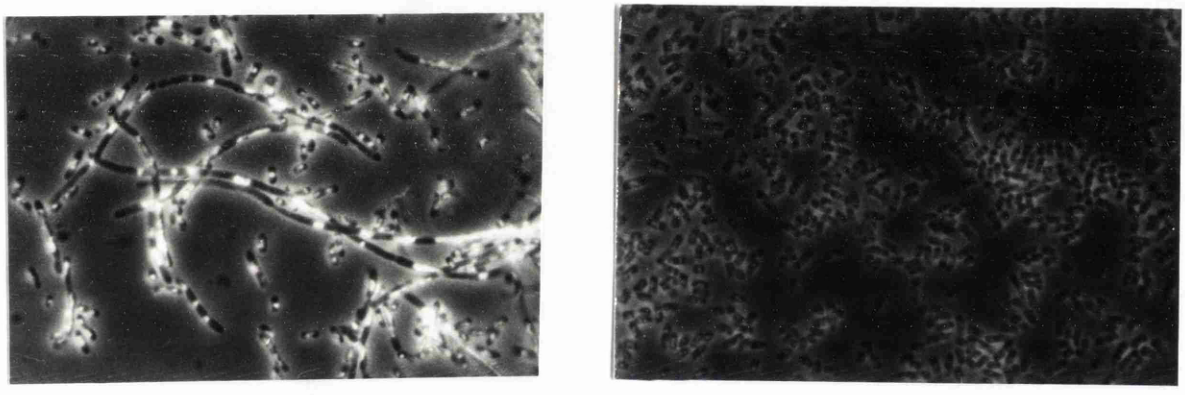


Figure 5.7. Verification of strains NL208 and NL202. Genomic DNA was prepared from DS941 (*dif*$^+$), NL40 (*difΔ6*), NL202 (*difΔ6 lacZ::loxP*) and NL208 (*difΔ6::loxP*) and cut with *Pvu*II. DNA was separated on a 1% agarose gel, and transferred to a synthetic membrane. Probing of this membrane with a radio-labelled *loxP* sequence gave the hybridisation seen with autoradiograph A, seen above. The membrane was then stripped of hybridised probe, and re-probed with a *dif* fragment, to give the hybridisation seen in autoradiograph B (transparent overlay above). This confirms that NL202 and NL208 have a deletion of the *dif* site, and that each contains a *loxP* sequence on a fragment of the size predicted by the physical map of the *E. coli* chromosome for the position of insertion (Kohara *et al.*, 1987).

Unlabelled lanes contain samples of no relevance to the experiments described.



A - NL208 **B - NL208 + Cre**

Fig. 5.8. Suppression of the *dif/xer* phenotype by the *loxP*/Cre system of bacteriophage P1. Exponential phase cultures grown in LB broth at 37° were prepared for photography by condensation and staining of cell nucleoids with chloramphenicol and DAPI respectively (as described in Chapter 3 and Materials and Methods). (**A**) NL208 (*loxP* site in place of *dif*) (**B**) NL208 + pRH200 (Cre expression vector). The long axis of each photograph represents approximately 70 μm. Condensation of the nucleoids does not interfere significantly with the phenotype demonstrated.

pN78. The results of these integration assays are shown in Table 4.2 and Fig. 7.4 and will be discussed below.

NL208 was transformed with pUC18, pSD105 (an XerC expression plasmid) and pRH200. NL40 (standard *dif* deletion) was also transformed with pRH200. The cell morphology of NL208 and of these transformants was investigated by microscopic analysis of untreated cultures, and after the staining of cell nucleoids with DAPI (Fig. 5.8). NL208 displays the characteristic cell morphology phenotype of *dif*/Xer mutants. This phenotype is unaffected by transformation with pUC18 or pSD105. However, transformation of NL208 with pRH200 fully suppresses this mutant phenotype. The phenotype of NL40 is unaffected by pRH200. Therefore, the action of the *loxP*/Cre recombination system is able to functionally replace the *dif*/Xer system in the replication terminus region.

5.5 Discussion

The results from experiments investigating the ability of other recombination systems to functionally replace *dif* are summarised in Table 5.2. The ability of the distantly related *loxP*/Cre recombination system to functionally replace *dif* is good evidence that the function of *dif* is really to provide site-specific recombination. These two recombination systems do both originate from actively partitioned replicons. However, *loxP* and *dif* share no significant sequence identity, although both sequences are quite AT-rich. Suppression by *loxP* also requires the Cre recombinase, which, although also an integrase family member, has very little sequence identity to XerC or XerD. Sequence comparisons between *dif* and *loxP* and between XerC, XerD and Cre are shown in Figs. 6.1, 1.2

Recombination system	Resolution selectivity	Plasmid integration	Phenotype
Tn*3 res* / Resolvase	YES	nd	Filamentous
cer / Xer	YES	nd	Filamentous
cer6 / Xer			
Accessory +	YES	NO	Filamentous
Accessory -	NO	YES	Filamentous
Phage P1 *loxP* / Cre	NO	YES	Wild-type
dif/Xer	NO	YES	Wild-type*

Table 5.2. Summary - Can other recombination systems suppress the *dif* phenotype? Other well charaterised site-specific recombination sites were introduced into the replication terminus of the *E. coli* chromosome, replacing *dif*. The corresponding recombinase enzymes were expressed in the cells, and the phenotype assayed. That recombinases and introduced sites were active for recombination was verified by plasmid integration assays. The observed phenotypes are shown. In the description of the *cer6* recombination system, 'Accessory +' refers to experiments conducted in a wild-type genetic background, whereas 'Accessory -' refers to experiments in an *argR* or *pepA* background. * - Introduction of a *dif* site into the terminus region generally resulted in a wild-type phenotype, however, exceptions to this are discussed in Chapter 4. 'nd' refers to experimental results not determined.

and 1.8. As discussed below a mechanism of functional replacement independent of recombination seems very improbable.

The *loxP*/Cre recombination system has been well characterised *in vitro*, and shown able to catalyse reactions between sites in any configuration (fusion, resolution or inversion reactions). The same is true of *dif* recombination when studied in plasmid substrates *in vivo* (Blakely *et al.* 1991). Although more recent work has suggested that *loxP*/Cre recombination shows some resolution selectivity *in vivo* (Adams *et al.*, 1992), any mechanism enforcing this selectivity is unlike that of the *res*/resolvase and *cer*/Xer recombination systems. This possible mechanism of *loxP*/Cre selectivity does not interfere with recombination between sites in other configurations *in vitro*, does not require flanking sequences and operates through an entirely unknown mechanism. There is strong evidence supporting a hypothesis that recombination at *dif* does not display resolution selectivity in the manner of other recombination systems (Blakely *et al.*, 1991, Chapter 4). If the function of the *dif*/Xer system is to resolve chromosome dimers, some other mechanism must ensure that recombination causes chromosome monomerisation not multimerisation. Therefore this mechanism must also be able to function with recombination being carried out by the *loxP*/Cre system.

It has been suggested that wild-type *dif* recombination could proceed in a rapid and unconstrained manner between sites on replicated chromosomes as they are actively separated, and that monomerisation would eventually be ensured by physical separation of the two copies of *dif* (Blakely *et al.*, 1991; Kuempel *et al*, 1991). Functional replacement of *dif* by *loxP* might be expected in this model. However, another model for chromosome resolution by *dif* has been proposed in which all chromosomes (whether monomeric or dimeric) normally undergo a single Xer-catalysed strand exchange to form a chromosomal Holliday

junction structure (Sherratt *et al.* 1993). This hypothesis is logical, as integrase family recombination is believed to pass through a short-lived Holliday junction intermediate (Hoess *et al.*, 1987; Nunes-Düby *et al.*, 1987; Jayaram *et al.*, 1988). Moreover, it has been shown that Xer recombination at *cer* can generate large quantities of Holliday junction structures both *in vivo* and *in vitro* (McCulloch *et al.*, 1994, and R. McCulloch and S. Colloms, unpublished work), although these structures have not been detected in recombination at *dif*. This alternative hypothesis cannot be discarded, as Cre is also a member of the lambda integrase family of recombinases, and the mechanism of catalysis at *loxP* is thought to proceed via a Holliday junction intermediate (Hoess *et al.*, 1987). However, there is evidence to suggest differences between recombination at *loxP* and *dif*. Specifically, in Xer recombination at both *cer* and *dif*, each recombinase protein seems to catalyse the exchange of a specific pair of DNA strands (Blakely *et al.*, 1993; McCulloch *et al.*, 1994; L. Arciszewska and G. Blakely unpublished work). In the single recombinase system, Cre carries out both pairs of strand exchange reactions, and does not have the possibilities for the regulation of recombination that may be present in the Xer system. These differences between the two recombination systems might be expected to interfere with the presumed controlled production and resolution of a chromosomal Holliday junction structure in this model for chromosome resolution. This controlled specificity of strand exchange would be crucial in this model, as once a chromosomal Holliday structure was formed, resolution to give monomeric products would require the exchanging of the correct pair of DNA strands. This controlled resolution would require information concerning the topological configuration of chromosomal DNA that might again be expected to rely upon chromosome partition mechanisms. This hypothesis could be tested by investigating the ability to suppress the *dif* phenotype of a non-selective recombination system known to

proceed without a Holliday junction intermediate (e.g. recombination at *gix* catalysed by Fis-independent mutants of Gin (Klippel *et al.*, 1988, Crisona *et al.*, 1994).

Neither the Tn3 *res*/resolvase, nor the *cer*/Xer recombination systems were able to suppress the *dif* deletion phenotype. If the functioning of *dif* relies upon the production of a Holliday junction, *res* would not be expected to functionally replace *dif* in the chromosome, as there is no evidence that resolvase recombination forms a Holliday junction intermediate (Grindley, 1994).

As previously described, there is good evidence that the function of *dif* is to provide recombination (Chapters 5 and 6). Work on the mechanism of Tn3 resolvase recombination would argue that if, as seems likely, *dif* functions through recombination between sites in chromosome multimers, or indeed on separate molecules, mechanisms enforcing selectivity on recombination at *res* would interfere with its ability to provide the recombination required for normal chromosome segregation and cell division. Models for resolvase recombination would predict that the system is unable to function in place of *dif* because recombination will not be catalysed by resolvase between sites that are 4.7 Mb apart on one DNA molecule, or on separate molecules. The biological role of this system in the transposon Tn3 is to resolve donor/target co-integrate molecules formed by replicative transposition, into two DNA molecules, each carrying a copy of the transposon (Arthur and Sherratt, 1979; Sherratt, 1989). Transposition of Tn3 displays transpositional immunity (Lee *et al.*, 1983, Sherratt, 1989); a transposon will not normally transpose into another part of the molecule that it is in, nor any other DNA molecule that already carries a copy of the same transposon. Therefore, transposition would not be expected to normally occur between two bacterial chromosomes, but between extra-chromosomal DNA.

Indeed, it has been reported that Tn3 does not efficiently transpose into the chromosome of a bacterial host (Sherratt, 1989). Therefore, the two copies of *res* in a co-integrate molecule would not normally be separated by more than the size of a large natural plasmid (50 - 100 kilobases of DNA). It has been proposed that the resolution selectivity of the resolvase system is enforced by a requirement for *res* site DNA and resolvase protein molecules to form a highly ordered synaptic complex in order for recombination to proceed. This requirement for a complex geometrical organisation of the recombinational synapse and wrapping of the DNA is believed to effect the topology and supercoiling of the DNA substrate, ensuring that synapsis and recombination are only energetically favourable if paired sites are in the correct configuration (the two sites being in direct repeat within the same DNA molecule). This mechanism would cause recombination to be energetically favourable only if sites are separated by the relatively short lengths of DNA described, able to wrap into the synaptic configuration (Stark *et al.*, 1989a, 1989b, Stark and Boocock, 1995). These ideas for the enforcement of selectivity are also discussed in the General Introduction, and Fig. 1.6.

The above hypothesis for resolvase selectivity suggests that the Tn3 *res*/resolvase system cannot functionally replace *dif* because of a selectivity mechanism limiting its recombination. A mechanism enforcing selectivity on *cer* recombination might rely on the same principles of substrate energetics as have been proposed for resolvase recombination. If so, it would be expected that *cer* fails to functionally replace *dif* for the same reasons as *res*. Recent experiments supporting this idea have shown that selective Xer recombination at the *psi* site of pSC101, which is closely related to *cer*, gives rise to a topologically specific product, a four noded catenane, and that Xer recombination at *cer* gives rise to a topologically specific Holliday junction (S. Colloms and D.

Sherratt personal communication). This strongly implies that selectivity at *cer* and *psi*, like *res*, is topological in nature. Indeed, the topology of the observed specific products appears to agree with the hypothesis that selectivity at *cer* and *psi* is mediated by the same substrate synapse topology and energetic principles as with *res*, but that strand exchange follows the topological path suggested for the integrase family recombinases rather than that for the resolvase family (Stark *et al.*, 1989a; S. Colloms, D. Sherratt and M. Stark personal communication).

However, it should be borne in mind that selectivity at *cer* and *res* may not share a similar mechanism, or even underlying principle. There are many differences between the two systems, with respect to both recombination mechanisms, and the means by which selectivity may be enforced. Tn3 resolvase and the Xer proteins are from different families of recombinase enzymes, the resolvase/invertase, and the integrase families respectively, believed to catalyse recombination by quite different mechanisms.

Introduction of an active *cer6* recombination site (the type II hybrid of Summers, 1989) into the terminus region in place of *dif* failed to suppress the *dif* deletion phenotype in any genetic background. As just discussed, known mechanisms of selectivity might interfere with the recombination required to fulfil the function of *dif*. Experiments looking at the *cer6* dependent integration of plasmids into the chromosome show that when inserted into the terminus region in place of *dif*, *cer6* behaves as it does in plasmid substrates (Summers, 1989). In the presence of the accessory proteins ArgR and PepA, recombination appears to show selectivity, intermolecular integration events being rare. However, in strains deficient in either ArgR or PepA, recombination appears to become non-selective, with a high frequency of integration events being detected. The failure to find functional replacement of *dif* by a *cer6* site that appears to show resolution selectivity would be predicted. However, why does *cer6* fail to

functionally replace *dif* in an *argR* or *pepA* background, in which it appears to display recombinational properties almost identical to *dif*? The reasons for this are very unclear.

As discussed in Chapter 4, aspects of the local sequence context around *dif* appear to effect its functioning. A similar phenomenon could be effecting recombination at *cer6* in the constructs used in this study. Although, the *cer6* site in the *difΔ6*::*cer6*Gm^R mutation used has a gross sequence organisation in which an introduced *dif* core site sequence is able to allow normal chromosome segregation (Figs. 4.5, 5.1), unlike the 33 bp *dif* core site, the *cer6* core site contains flanking DNA on both sides. Thus, other factors of the local sequence context, for example transcription from the P_{cer} promoter located within the *cer6* sequence (Summers and Sherratt, 1988; Patient and Summers, 1993), could have an effect. Also, the DNA flanking the *cer6* core site on the side derived from the original cloning of the CloDF13 *parB* site (Hakkaart *et al.*, 1984) is very poorly characterised. As mentioned in Chapter 4, investigation of any predicted stable structures fomed by the DNA around the *cer6* site in these strains would be useful in addressing these issues. It should be borne in mind that results from Chapter 4 could be interpreted as suggesting that very small perturbations in the recombination efficiency of chromosomal sites could interfere with the *dif* function. However, the possibility remains that for the wild-type functioning of *dif* in chromosome segregation, more is required than efficient non-selective recombination, such as the assumption of a particular DNA configuration around the recombination site allowing the determination of the DNA topology of a chromosomal Holliday structure (see Holliday junction model for *dif* function above) by a resolving enzyme complex. Such a hypothesis would require that *dif*/Xer recombination and *loxP*/Cre recombination share important characteristics not shared with non-selective *cer6*//Xer recombination, and

might be testable by constructing strains using only the *cer6* minimal site in place of *dif*.

It has been shown that when mechanisms of selectivity are not acting upon it, *cer6* has very similar recombinational properties as *dif* in *in vivo* recombination assays, and in *in vitro* assays looking at the binding of XerC and XerD to DNA (Summers, 1989; G. Blakely, personal communication). However, experiments looking at strand cleavage of suicide recombination substrates *in vitro* have failed to detect 'bottom' strand cleavage of *cer6*, whereas this is clearly detectable, catalysed by XerD, using *dif* and *psi* (G. Blakely, personal communication). Again, the functional significance of this result is unclear.

Cornet *et al.*, (1994) report that the Xer recombination site *psi* of pSC101 shows different recombinational properties when in the bacterial chromosome and on plasmids. When in the chromosome, it appears able to functionally replace *dif* , through unconstrained (non-selective) recombination, not requiring accessory sequences, whereas in plasmid substrates, recombination is selective, and requires accessory sequences and proteins. Since *psi* and *cer* have significant homology, and show similar properties in *in vivo* recombination assays in plasmid substrates, (and *in vitro* with the exception that *cer* produces Holliday junctions not recombinant products, S. Colloms, M. Burke and D. Sherratt personal communication), this might suggest that *cer* and other sites that display recombinational selectivity in plasmid substrates, such as *res*, could also functionally replace *dif*. However, if any recombination sites were, for reasons unknown, to function differently on the chromosome than on plasmid substrates, this phenomenon might be expected to be limited to Xer recombination, and indeed, *in vitro* recombination studies have shown *psi* to have different properties to other Xer recombination sites (S. Colloms and D. Sherratt unpublished work).

Chapter 6

The Xer recombinases and *dif* function.

Introduction - The Xer recombinases and *dif* function.

XerC and XerD are members of the lambda integrase family of site-specific recombinases (Colloms *et al.*, 1990; Blakely *et al.*, 1993). It has been shown that both proteins are required for recombination at *dif* and *cer*, and for normal chromosome segregation (Blakely *et al.*, 1991, 1993; Chapter 3). Is their function in chromosome segregation to provide recombination?

6.1 The phenotypic effect of mutating putative active site residues of XerC and XerD.

The lambda integrase family of site-specific recombinases share two highly conserved regions in their protein sequences, Domains I and II (Argos *et al.*, 1986; Blakely *et al.*, 1993). Within these domains are found four completely conserved residues implicated in catalysis (Argos *et al.*, 1986; Pargellis *et al.*, 1988; Parsons *et al.*, 1988; Abremski and Hoess, 1992; Figs. 1.7 and 6.1). In both XerC and XerD, two of these residues were mutated by site-directed mutagenesis (Blakely *et al.*, 1993). The putative active site tyrosine, shown in other systems to be the attacking nucleophile in strand exchange (Gronostajski and Sadowski, 1985; Pargellis *et al.*, 1988; Evans *et al.*, 1990), was converted to a phenylalanine, and the domain II conserved arginine was converted to a glutamine residue. Results from the related Flp and Cre recombination systems implicate this conserved arginine in DNA binding and phosphodiester activation (Parsons *et al.*, 1990, Abremski and Hoess, 1992). In single recombinase lambda integrase systems (the λ Int, Flp and Cre systems) these changes are known to abolish recombination entirely (Prasad *et al.*, 1987; Pargellis *et al.*, 1988; Parsons *et al.*, 1988, Abremski and Hoess, 1992). Blakely *et al.*, (1993) analysed the mutant proteins XerCY275F,

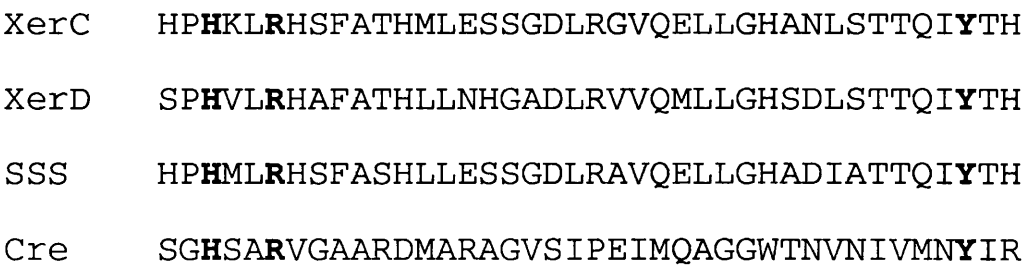


Figure 6.1. Amino acid sequence alignment of the conserved domain two regions of XerC, XerD, SSS, and Cre. This figure shows sequence alignments from four lambda integrase family proteins XerC, XerD, SSS (an XerC homolog from *Pseudomonas aeruginosa*) and the Cre recombinase of bacteriophage P1 (Argos *et al.*, 1986; Blakely *et al.*, 1993; Höfte *et al.*, in press). The conserved domain two regions of each protein are shown, highlighting in bold face the three residues of this domain conserved within all members of the integrase family of recombinases. The effects of mutating the conserved arginine and tyrosine within this domain are described in Chapter 6. An alignment of the complete amino acid sequence of XerC and XerD is shown in Fig. 1.7. The amino acid sequence of Cre does not have sufficient homology to the Xer proteins to produce a convincing alignment of the entire protein sequence.

XerDY279F, XerCR243Q and XerDR247Q individually for their ability to catalyse *in vivo* recombination of plasmid substrates in the presence of a wild-type copy of the other recombinase. All four mutant proteins were found to bind *dif* DNA *in vitro*. Although mutants of XerC were found to be completely unable to support Xer-mediated recombination, low levels of recombination were detected when mutant XerD proteins were combined with wild-type XerC, resolution of reporter plasmids being at greatly reduced rates. It was suggested that this low level of recombination is caused by the action of Holliday junction resolving enzymes (possibly including XerC) upon Holliday junctions formed by an XerC catalysed first strand exchange (Blakely *et al.*, 1993).

Can these mutant recombinases function like wild-type XerC and XerD in normal chromosome segregation? The hypothesis that *dif* is a chromosomal resolution site would predict otherwise. To test this, the phenotype of strains with mutations in chromosomal Xer genes expressing the corresponding mutant Xer protein from high copy number plasmids was tested.

6.2 XerC mutants XerCY275F and XerCR243Q

Plasmids expressing mutant derivatives of XerC were based on the XerC expression vector pSD104, which is pTZ18R (Pharmacia) *xerC*$^+$ (Colloms *et al.*, 1990). pLA121 expresses XerCY275F, and pLA130 expresses XerCR243Q (Blakely *et al.*, 1993). These plasmids were transformed into DS984, a strain whose chromosomal *xerC* gene contains a mini-Mu insertion known to abolish XerC recombinase activity (Colloms *et al.*, 1990). After transformation, these strains were grown twice over-night on solid media containing ampicillin, and observed untreated using the microscope. The phenotypes consistently observed are detailed in the following Table 6.1.

Table 6.1

Protein expressed in DS984 and plasmid	Apparent phenotype
XerC (pSD104)	Wild-type
XerCY275F (pLA121)	Filamentous
XerCR243Q (pLA130)	Filamentous
XerD (pRM130)	Filamentous

These XerC expression plasmids did not display the instability of the XerD vectors (see below).

6.3 XerD mutants XerDY279F and XerDR247Q

Previous work has shown some Xer recombination in cells expressing these mutant XerD proteins, although the plasmid expressing XerDY279F in these experiments, pLA123, was shown later to carry a secondary mutation *XerDE184K* (Blakely *et al.*, 1993). Therefore it was decided to analyse further the recombination occurring in the presence of these proteins, as well as the phenotype of cells expressing them. Plasmids have been produced and sequenced, that should express each mutant XerD protein with only a single amino acid residue changed (L. Arciszewska unpublished work). Plasmids expressing mutant XerD proteins are all based on the XerD expression vector pRM130, which is pUC19 *xerD*$^+$ (Blakely *et al.*, 1993). pLA127 expresses XerDR247Q, pLA139 expresses XerDE184K, and pLA137, pLA137Y and pLA137A are independently isolated plasmids expressing XerDY279F. DS9008

(*xerC*$^+$ *xerD* mini-Tn *10* insertion) was transformed with each XerD expression plasmid and, as a control, the XerC expression plasmid pSD105.

During these experiments, it became evident that the XerD expression plasmid pRM130 and plasmids derived from it, expressing mutant proteins, are very unstable. Plasmid DNA was found to be lost from cells during overnight growth, despite the initial presence of high levels of ampicillin (100 μg/ml) in growth media. Although this greatly interfered with experiments using these plasmids, it seemed interesting and so attempts were made to quantitate this instability. Therefore, the fraction of cells retaining ampicillin resistance after growth in liquid culture with antibiotic was estimated. Plasmid-containing DS9008 cells were grown overnight on LB agar + 100 μg/ml ampicillin, then a few cells inoculated into fresh LB broth with ampicillin and shaken at 37° for 5 hours. Cultures were then diluted and plated onto LB plates with and without 100 μg/ml ampicillin. The numbers of colonies for different plasmids are given in Table 6.2. The XerC expression vector used as a control, pSD105 (Colloms *et al.*, 1990) is pBAD *xerC*$^+$.

Table 6.2

Plasmid and protein expressed	**No. of ampicillin resistant colonies**	**No. of colonies (no antibiotic)**
pSD105 (XerC)	≈700	≈700
pRM130 (XerD)	11	547
pLA137Y (XerDY279F)	3	≈1000
pLA139 (XerDE184K)	10	≈650

The instability of these plasmids is clear, as plasmid loss occurs despite high initial concentrations of antibiotic. Although this rough quantification was carried out with an *xerD* mutant host, instability also appears evident in wild-type cells. The reason for this instability remains unclear.

Analysis of the effects of mutant XerD proteins was made easier by growing cells on solid media containing high levels of ampicillin and 1% glucose. Plasmid instability seemed less severe when cells were grown on solid media. Due to the apparent depletion of antibiotic in liquid cultures (presumably through the activity of beta-lactamase), it might be expected that the exposure of cells to antibiotic would be longer lasting for cells grown on plates, as diffusion of antibiotic into colonies, and beta-lactamase enzyme away, would be slowed, allowing some antibiotic to persist for longer periods of time. Growth of cells in the presence of 1% glucose was also found to greatly increase plasmid stability. This would be expected to act by reducing the expression of cloned genes from the lac promoter of these pUC19 derived plasmids. It was only with growth on glucose that the wild-type XerD expression vector pRM130 would consistently fully suppress the filamentous phenotype of the *xerD* mutant DS9008. Therefore, at least with the wild-type XerD expression plasmid, the repression of expression caused by growth on glucose does not affect the functioning of the protein.

6.4 Recombination catalysed by mutant XerD proteins.

A reporter plasmid assay (described in Chapter 4 and Fig. 4.1), with the *cer* reporter plasmid pCS202 (Colloms *et al.*, 1990) was used to investigate Xer recombination catalysed by these mutant XerD proteins. pCS202 is a λdv-based plasmid containing two *cer* sites in direct repeat flanking a tetracycline resistance gene. Active Xer recombination therefore deletes this marker, and

can be detected by selection or by visualisation of DNA on agarose gels. This reporter plasmid was used to analyse recombination catalysed by wild-type XerC from the chromosomal gene, and the mutant XerD proteins encoded by the expression plasmids. pCS202, a *cer* reporter plasmid, was used because all *dif* reporter plasmids constructed and available use the same ColE1 plasmid origin of replication as the mutant protein expression plasmids. It also seemed extremely unlikely that the role of, and requirement for, putative active site residues of the Xer proteins during recombination would differ between *cer* and *dif*.

To perform this reporter plasmid assay, transformants including each expression vector were further transformed with pCS202. The extent of Xer mediated deletion of the tetracycline resistance gene from pCS202 (to give pCS203) was estimated at various times after transformation by analysis of plasmid DNA on agarose gels and by selection on tetracycline. DNA was prepared from cells transformed with pCS202 and expression plasmids after different numbers of generations growing on LB media with 100 μg/ml ampicillin, 25 μg/ml chloramphenicol and 1% glucose. Samples were analysed by separation on 1% agarose gels (Fig. 6.2 and data not shown). The last DNA samples, prepared approximately 80 generations after transformation, were also analysed for tetracycline resistance, by transformation into competent DS9008 and selection on chloramphenicol and on tetracycline. The numbers of transformants recovered with each antibiotic is given in Table 6.3

Expression plasmid with pCS202 for 80 generations in DS9008	No. of tetracycline resistant colonies	No. of chloramphenicol resistant colonies
pRM130 (*XerD*$^+$)	0	862
pLA127 (*XerDR247Q*)	63	146
pLA137Y (*XerDY279F*)	0	122
pLA137 (*XerDY279F*)	11	140
pLA137A (*XerDY279F*))	12	60
pLA139 (*XerDE184K*)	3	77
pSD105 (*xerC*$^+$)	103	365
unresolved pCS202	409	945
no pCS202	0	0

Table 6.3 "unresolved pCS202" refers to plasmid DNA originally prepared from an *xer* mutant strain and not passaged through an experimental host prior to transformation to assay for comparative resistance to chloramphenicol and tetracycline. This result would be expected to reflect differences in the ability of plasmid borne chloramphenicol and tetracycline resistance genes to allow the formation of a viable antibiotic resistant colony. The differences between results for each plasmid expressing XerDY279F in this data and in Fig. 6.2 were not reproducible between experiments and should not be taken to indicate a significant difference between these plasmids.

Although this data shows no detectable breakdown of pCS202 in cells expressing XerDR247Q, plasmids expressing XerDY279F consistently allow recombination at levels above those seen with vectors expressing XerC or

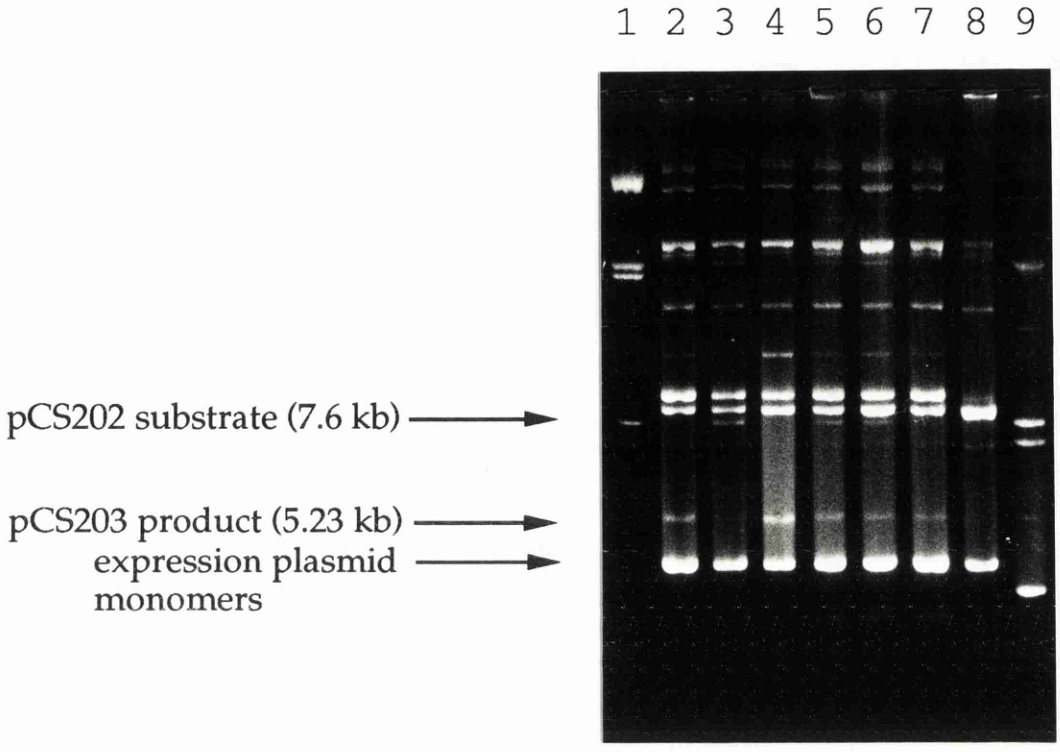


Figure 6.2. Xer recombination mediated by mutant XerD proteins. The *cer* reporter plasmid pCS202 was used to analyse Xer recombination mediated by mutant XerD proteins. DS9008 (*xerD*) was transformed with plasmids expressing each mutant protein to be tested (see main text for details) and subsequently with pCS202. DNA was prepared after approximately 80 generations of growth on antibiotics and 1% glucose, and analysed by 1% agarose gel electrophoresis. Samples in each lane were as follows,

1. pCS202 marker
2. DNA prepared from DS9008 + pCS202 + pRM130 (wild-type XerD)
3. DNA prepared from DS9008 + pCS202 + pLA127 (XerDR247Q)
4. DNA prepared from DS9008 + pCS202 + pLA137Y (XerDY279F)
5. DNA prepared from DS9008 + pCS202 + pLA137 (XerDY279F)
6. DNA prepared from DS9008 + pCS202 + pLA137A (XerDY279F)
7. DNA prepared from DS9008 + pCS202 + pLA139 (XerDE184K)
8. pLA139 marker
9. DNA prepared from DS9008 + pCS202 + pSD105 (wild-type XerC)

The protein expressed from each plasmid is detailed. A strain containing the XerC expression vector pSD105 was used as a control. pSD105 is smaller than the XerD expression plasmids used. These results are discussed in the main text.

XerDR247Q. XerDE184K does not behave very differently from wild-type XerD in these assays.

Blakely *et al.*, (1993) propose that low levels of recombinant products seen in the presence of mutant XerD proteins are caused by the formation of a Holliday junction structure by an XerC catalysed strand exchange, followed by low efficiency resolution of this structure to give products. The formation of large quantities of such Holliday structures by *cer* recombination has been established *in vivo* and *in vitro* (McCulloch *et al.*, 1994 and R. McCulloch unpublished). More recent work on *in vitro* recombination would also support the view that this first strand exchange producing Holliday junctions is catalysed by XerC (L. Arciszewska and G. Blakely unpublished). This hypothesis would explain the products seen in the experiments presented here in the presence of XerDY279F. However, there seem to be two possible reasons why no products were seen with XerDR247Q. Firstly, XerDR247Q may be unable to support the first XerC catalysed strand exchange to give a Holliday junction in these *in vivo* experiments. This seems unlikely, as in experiments almost identical to those presented here, Blakely *et al.*, (1993) saw a similar low level of recombinant products as with XerDY279F. Experiments performed *in vitro* using a Holliday junction substrate closely related to *cer* also show XerC mediated strand exchange supported by cell extracts containing XerDR247Q (L. Arciszewka unpublished). Secondly it is possible that there is insufficient activity of XerDR247Q in these experiments to support detectable levels of recombination. This could be due to instability of the expression vector pLA127, insufficient expression of active protein from this plasmid, perhaps caused by mutation in the plasmid isolate used, or other causes. Since results with pLA127 are in contrast to those with otherwise identical plasmids expressing other XerD mutant proteins, any problem with protein activity would need to be specific to this plasmid. As discussed, plasmid instability is a potential problem with the XerD expression

plasmids used. However, there is no evidence of plasmid instability when host cells were grown on glucose, as large amounts of plasmid DNA are visible in all DNA preparations from these cells, and plasmids expressing other XerD proteins are sufficiently stable to allow XerD activity. This lack of XerDR247Q activity might be expected from experiments looking at binding of mutant XerC and XerD proteins to *dif* DNA (Blakely *et al.*, 1993). Assaying the binding activity of cell extracts to *dif* DNA, extracts from cells expressing XerDR247Q showed significantly less binding than extracts from cells expressing other mutant Xer proteins (L. Arciszewska personal communication). Whether this lack of binding activity is due to lack of protein expression, protein instability, poor binding of the protein to DNA, or other reasons is not known.

Another explanation for the low levels of recombination seen in cells expressing mutant XerD proteins could be the production of a wild-type *xerD* gene by homologous recombination between the mutant *xerD2-Tn10-9* gene in the chromosome and the *xerDY279F* gene on plasmids. However, this might be expected to cause a fully wild-type level of recombination (and phenotype, see below), as the growth advantage over a filamentous *xerD* strain might be expected to select for a wild-type allele in the lengthy duration of these experiments. This also seems unlikely as it would need to have occurred in several different experiments in plasmids expressing XerDY279F, but never XerDR247Q.

6.5 The phenotype of cells expressing mutant XerD proteins

The phenotype of these strains expressing mutant XerD proteins growing on glucose was investigated by microscopic analysis of both untreated cultures and those stained with DAPI for visualisation of cell

nucleoids (Chapter 3). The results are presented in the following Table 6.4 and in Fig. 6.3.

Table 6.4

Protein expressed in DS9008 and plasmid	Apparent Phenotype
XerD (pRM130)	Wild-type
XerDR247Q (pLA127)	Filamentous
XerDY279F (pLA137Y)	Filamentous
XerDY279F (pLA137)	Filamentous
XerDY279F (pLA137A)	Filamentous
XerDE184K (pLA139)	Wild-type
XerC (pSD105)	Filamentous

6.6 Discussion

These data suggest that the putative active site tyrosine and the domain II conserved arginine of both XerC and XerD are all required for normal chromosome segregation in *E.coli*. The possible failure to express XerDR247Q in cells should be remembered. Since XerCY275F, XerCR243Q and XerDR247Q seem completely unable to take part in Xer recombination these results are not surprising. In light of other experiments looking at the requirements for site-specific recombination in the terminus, it is not surprising that the greatly reduced recombination seen in reporter plasmid assays with XerDY279F does not result in a wild-type phenotype. The inability of the *cer6* site to functionally substitute for *dif*, and the interference with *dif* function caused by factors of local DNA context (Chapters 4 and 5) seem to suggest that

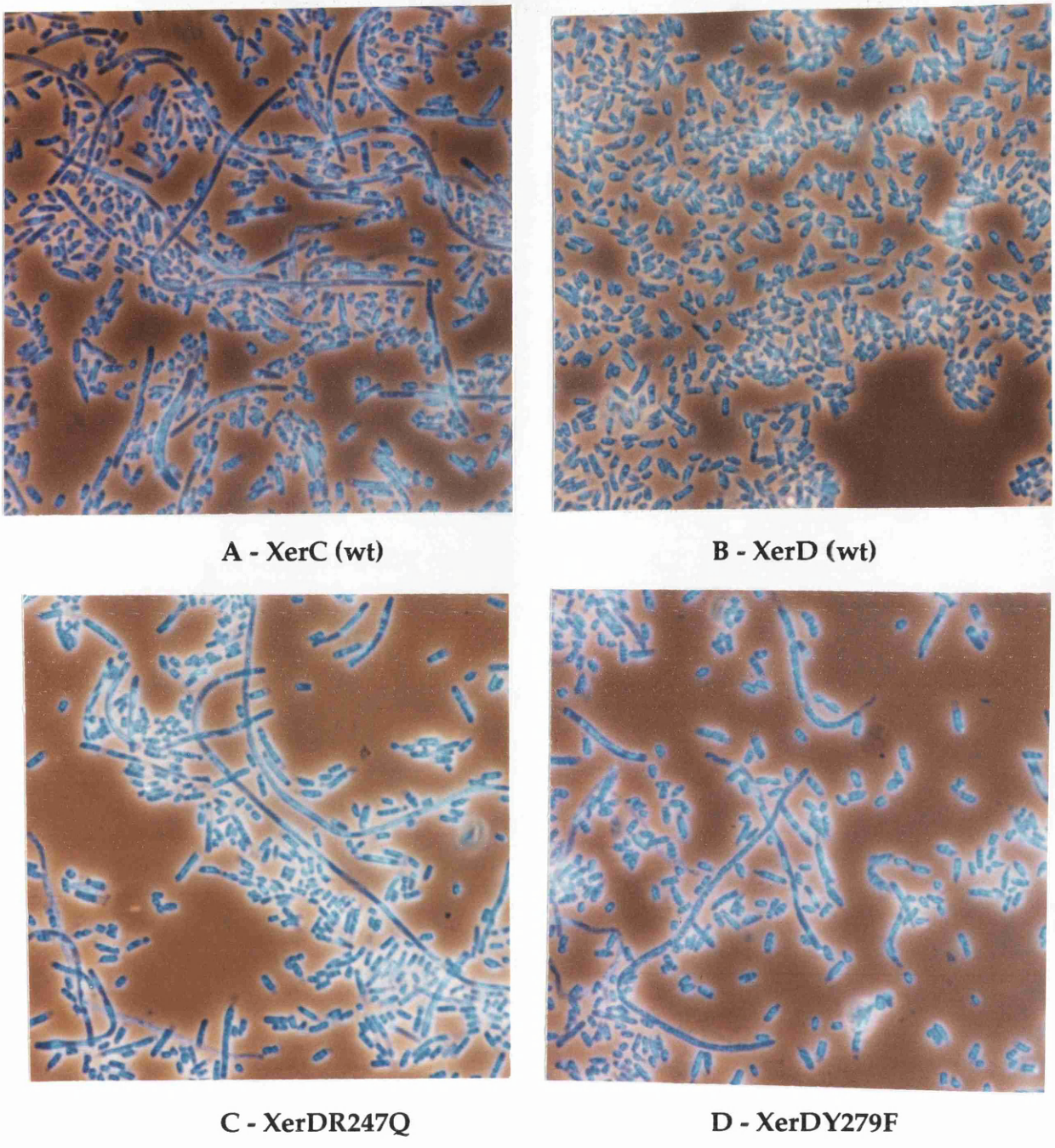


Figure 6.3. The cell and nucleoid morphology of cells expressing mutant XerD proteins. This figure shows the morphological phenotype of DS9008 (*xerD*) transformed with different XerC and XerD expression plasmids. Exponential phase cultures grown in LB broth at 37° with anibiotic and 1% glucose were prepared for photography by condensation and staining of cell nucleoids with chloramphenicol and DAPI respectively (Chapter 3 and Materials and Methods). (**A**) DS9008 + pSD105 (XerC expression plasmid) (**B**) DS9008 + pRM130 (Wild-type XerD expression plasmid) (**C**) DS9008 + pLA127 (XerDR247Q expression plasmid) (**D**) DS9008 + pLA137A (XerDY279F expression plasmid). The axes of each photograph represents approximately 70 μm. Condensation of the nucleoids does not interfere significantly with the phenotype demonstrated.

relatively little interference can have a large phenotypic effect. Since these particular residues of the Xer proteins are strongly implicated in the catalysis of recombination, and their mutagenesis should not greatly change other properties of the proteins, this data support the hypothesis that the biological function of *dif* and the Xer system is to provide site-specific recombination.

Chapter 7

The effect of chromosomal position on *dif* function

Introduction - The effect of chromosomal position on *dif* function

The circular *E.coli* chromosome is replicated bi-directionally. Two replication forks initiated at a specific chromosomal origin of replication, *oriC*, proceed in both directions and meet in the replication terminus region. *dif* is naturally located in this terminus region (Fig 7.1; Kuempel *et al.*, 1991). The replication termination system of *E.coli* consists of several (six have been recognised to date) *cis*-acting replication terminator or *Ter* sites located in and around the terminus region, and a *trans* acting protein Tus (reviewed by Kuempel *et al.*, 1989 and Hill, 1992). Each *Ter* site, when bound by Tus, acts as a block to the progress of replication forks approaching it from one side only. Replication forks approaching from the other direction are not affected. The chromosomal arrangement of these *Ter* sites is such that the termination system stops replication forks leaving the terminus region, and should ensure that the two chromosomal replication forks meet in the DNA (about 250 kb) between the innermost *Ter* sites *TerA* and *TerC*. *dif* is located in this DNA approximately 16 kb from *TerC* (Fig. 7.1). Surprisingly, no phenotype has yet been found to be caused by inactivation of the replication termination system by deletion of the *tus* gene.

Is the natural positioning of *dif* in the replication terminus region coincidental, or is this positioning required for its function? To investigate this, experiments were conducted to determine whether a *dif* site is able to fulfil its function if positioned elsewhere on the chromosome. Therefore strains were constructed in which *dif* had been deleted from its natural location in the replication terminus, and an ectopic *dif* site introduced elsewhere. It was decided to introduce ectopic sites close to the origin of replication, at minute 84, and approximately mid-way between the origin and the terminus, at minute 8. Near the origin, at minute 84, *dif* was inserted into

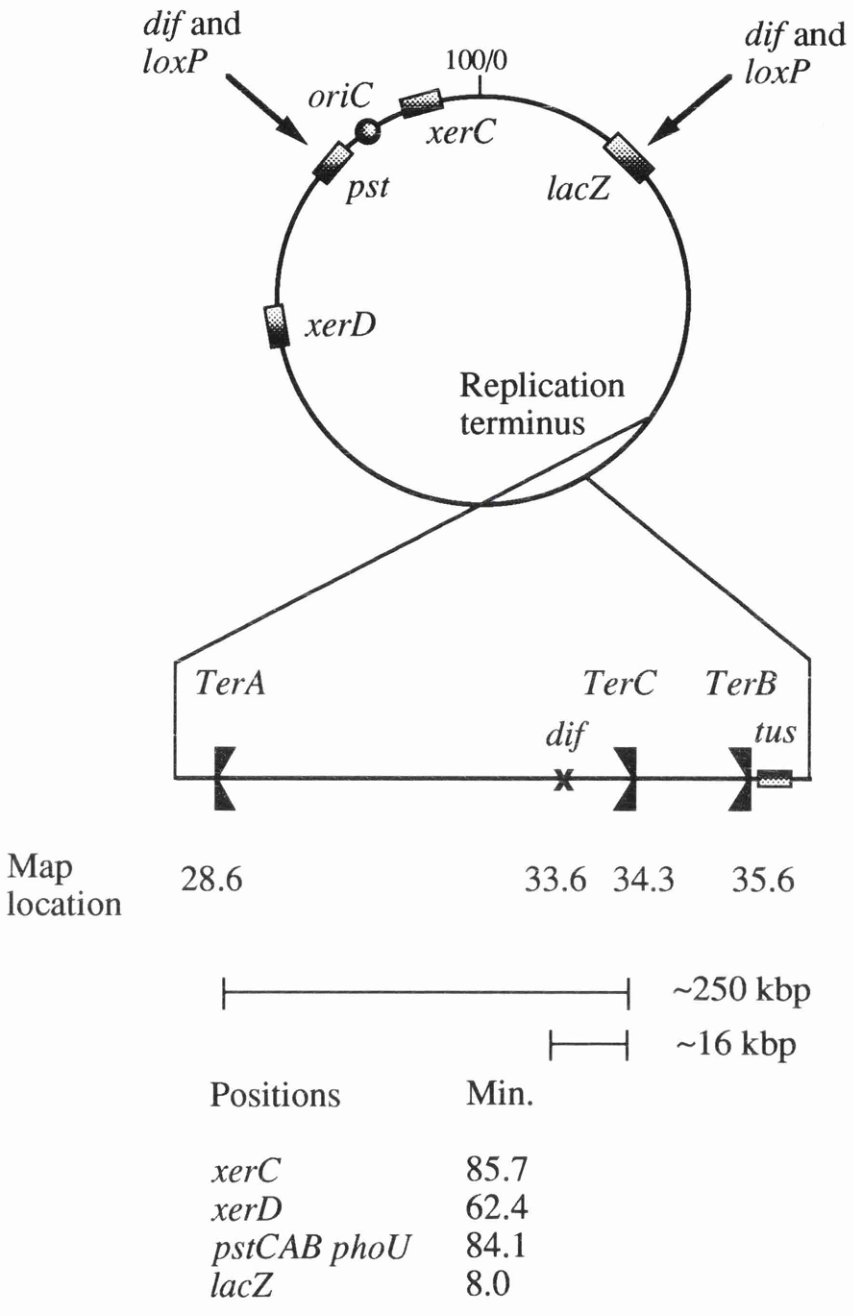


Figure 7.1. The *E. coli* chromosome - the replication terminus region and chromosomal locations used to study positional effects upon *dif* function. This representation of the *E. coli* chromosome shows *xerC*, *xerD* and the two locations into which ectopic recombination sites have been inserted, relative to the natural location of *dif* in the replication terminus, the position 0 minutes and *oriC*. Positions in minutes refer to the genetic linkage map of Bachmann, (1990). Three of the six identified chromosomal *Ter* sites are shown, represented such that replication forks approaching from the flat side are not affected (eg. *TerC* halts replication forks travelling from left to right).

a deletion of *pstC*, *pstA*, *pstB* and *phoU* genes, controlling phosphate metabolism, and at minute 8 into a small deletion within the *lacZ* gene, encoding beta-galactosidase (Fig. 7.1). These specific positions were chosen as they are well characterised and display easily detectable mutant phenotypes without affecting cell morphology.

33 bp *dif* core site sequences introduced into the terminus region in place of the wild-type *dif* locus did not always suppress the *dif* deletion phenotype, despite being active for site-specific recombination (Chapter 4). When larger *dif* fragments (532 bp, Fig. 4.3) were used, suppression was full and consistent. Therefore, for the introduction of *dif* elsewhere on the chromosome, a 532 bp *dif* fragment was used, and in a configuration, with respect to antibiotic resistance markers used in construction, that had always given full phenotypic suppression with *dif* in the terminus; the Gm^R 532 bp *dif* fragment from pN57 and NL287 (Fig. 4.5).

7.1 Insertion of *dif* into *lacZ* at minute 8 of the chromosome

dif was inserted into a small deletion within the *lacZ* gene at minute 8, kilobase 375 of the chromosome, approximately half way between *oriC* and the replication terminus region (Fig. 7.1). *lacZ* encodes beta-galactosidase, an enzyme of the lactose operon that naturally cleaves lactose, giving glucose and galactose. *lacZ* and beta-galactosidase are well known from their extensive use in molecular biology (Miller, 1992). p357 (M. Burke and D. Sherratt unpublished work) was used as a source of DNA from *lacZ*. This plasmid has a 3.1 kb restriction fragment containing the whole *lacZ* coding sequence cloned into pUC9. Restriction enzyme sites for manipulation of sequences were taken from the physical maps of *lacZ* and the whole

chromosome (Kohara *et al.*, 1987; Miller, 1992) and the nucleotide sequence of *lacZ* (Kalhins *et al.* 1983).

Insertion of the Gm^R 532 bp *dif*$^+$ fragment from pN57 (approximately 2 kb in size), and the Gm^R fragment of pN12 (≈1.5 kb), into a small deletion in the centre of the *lacZ* coding sequence in p357, gave pN37 (*lacZ*::532bp*dif*Gm^R) and pN32 (*lacZ*::Gm^R) respectively. These plasmids could then be used to introduce the gentamicin resistance marker into the chromosomal *lacZ* gene, with and without *dif*, by the linear transformation method used previously (Chapter 3). The possibility was considered that recombination between copies of *dif* located outside the terminus region might produce large numbers of chromosome multimers, and perhaps even be lethal to cells. Therefore, strains with *dif* outside the terminus region were originally produced carrying an *xerC* mutation. Thus the mutations *lacZ*::532bp*dif*Gm^R and *lacZ*::Gm^R were transferred into the chromosome of NL13 (AB1157 *recBCsbcBC difΔ6 xerC*) to give NL137 and NL132 respectively, and in turn transduced into NL50 (DS941 *difΔ6 xerC*::*y17*), to give NL237 and NL232 respectively.

7.2 Insertion of *dif* into the *pst* genes close to *oriC*

dif was inserted into a deletion of the genes *pstC, pstA, pstB* and *phoU* at minute 84, kilobase 3800 of the *E.coli* chromosome, approximately 20 kb away from *oriC*. These genes are involved in regulating genes of the phosphate regulon, and in the transport of inorganic phosphate (Wanner, 1986; Niedhart *et al.*, 1987). Specifically, strains mutant in these genes constitutively express alkaline phoshatase (AP) regardless of the level of phosphate in the surrounding media (Wanner and Latterell, 1980). The plasmid pSN518 (Amemura *et al.*, 1982) was used as a source of DNA from

this region. Information regarding restriction enzyme sites was taken from the physical maps of the *pst* genes and the whole chromosome (Amemura *et al.*, 1982; Kohara *et al.*, 1987) and the nucleotide sequence of this region (Amemura *et al.*, 1985).

The Gm^R 532 bp *dif*$^+$ fragment from pN57, and the Gm^R fragment from pN12 were each inserted into the more central of the two chromosomal deletions already present in pSN518 (both approximately 3 kb in size, Amemura *et al.*, 1982) to give pN47 (*ΔpstCABphoU*::532bp*dif*Gm^R) and pN42 (*ΔpstCABphoU*::Gm^R) respectively. These mutations on pN47 and pN42 were then transferred into the chromosome of NL13 by linear transformation, giving NL147 and NL142 respectively, and in turn transduced into NL50 (DS941 *difΔ6 xerC*::*y17*) to give NL347 and NL342 respectively.

7.3 The phenotype of strains containing *dif* outside the replication terminus region

The phenotypic effect of *dif* being located close to *oriC* or in *lacZ* in the *xerC*$^-$ strains NL347 and NL237 was assayed by transformation with an XerC expression plasmid and microscopic analysis. The strains NL290 (*difΔ6*::532bp*dif*Gm^R *xerC*), NL232, NL237, NL342 and NL347 were transformed with the expression vector pBAD and the XerC expression plasmid pSD105 (pBAD *xerC*$^+$, Colloms *et al.*, 1990).

Transformants were twice sub-cultured and grown overnight before assaying of their phenotype, in order that protein expression could be established from plasmids, and to allow time for normal cells to outgrow any filaments. The cell morphology of untreated cultures of these transformant strains was then analysed by simple microscopic observation. The phenotypes

consistently displayed are shown in the following Table 7.1. 'Filamentous' corresponds to the characteristic *dif/xer* cell morphology phenotype.

Table 7.1

Strain	Plasmid	Apparent phenotype
NL290 (*dif* in terminus)	pBAD (vector only)	Filamentous
	pSD105 (*xerC*$^+$)	Wild-type
NL237 (*dif* in *lacZ*)	pBAD	Filamentous
	pSD105	Filamentous
NL347 (*dif* in *pst*)	pBAD	Filamentous
	pSD105	Filamentous

Experimental control strains with only gentamicin resistance genes inserted in *lacZ or* in the *pst* genes, NL232, and NL342 respectively, also displayed a *dif*/Xer phenotype when transformed with either pBAD, or with pSD105. The expression of XerC in these strains had no noticeable effect on the colony size of strains with ectopic *dif* sites. All strains carrying deletions of the chromosomal *pst* genes produced smaller colonies, but this was in no way affected by *dif* or XerC.

These results suggest that Xer recombination at a *dif* site located outside the replication terminus is not lethal, nor has a great detrimental effect upon the growth rate of strains. Therefore, it should be possible to maintain these ectopic *dif* constructs in Xer$^+$ strains. However, in this experiment neither ectopic *dif* construct is able to suppress the phenotype caused by deletion of the wild-type *dif* locus, when XerC is expressed from a high copy-number plasmid. Would the same be true with wild-type expression of XerC? Therefore, *lacZ*::532bp*dif*Gm^R, *lacZ*::Gm^R, *ΔpstCABphoU*::532bp*dif*Gm^R and *ΔpstCABphoU*::Gm^R, were transduced into both NL40 (*difΔ6*) and DS941. This

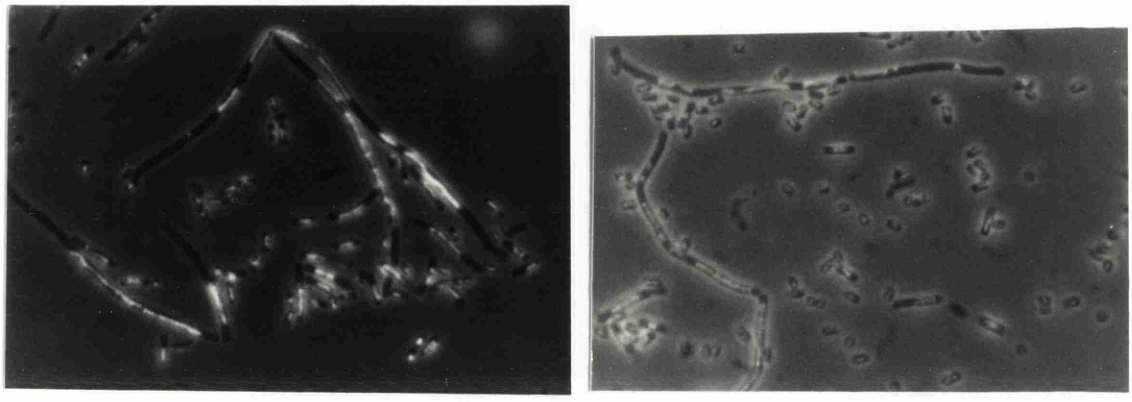
gave strains with wild-type expression of the Xer proteins and ectopic *dif* sites, with or without the original wild-type *dif* locus.

The cell morphology of these newly created mutant strains in untreated cultures was analysed by simple microscopic observation. The nucleoid morphology of NL350 (NL40 *ΔpstCABphoU*::532bp*dif*Gm^R) and NL250 (NL40 *lacZ*::532bp*dif*Gm^R) was investigated by staining with DAPI and observation using phase-contrast/fluorescence microscopy . The strains constructed and the resultant phenotypes are shown in the following Table 7.2, Fig. 7.2 and data not shown.

Table 7.2

Strain	**Genotype**	**Apparent phenotype**
DS941	wild-type	wild-type
NL40	*difΔ6*	filamentous
NL343	*ΔpstCABphoU*::Gm^R	wild-type
NL345	*difΔ6 ΔpstCABphoU*::Gm^R	filamentous
NL348	*ΔpstCABphoU*::532bp*dif*Gm^R	wild-type
NL350	*difΔ6 ΔpstCABphoU*::532bp*dif*Gm^R	filamentous
NL233	*lacZ*::Gm^R	wild-type
NL238	*lacZ*::532bp*dif*Gm^R	wild-type
NL250	*difΔ6 lacZ*::532bp*dif*Gm^R	filamentous

Experiments studying the suppression of the *dif* deletion phenotype by various *dif* containing sequences in the terminus region had suggested that the local sequence context of the site, possibly including the transcription of surrounding DNA, could effect the functioning of the site (Chapter 4). All strains constructed with an ectopic *dif* site introduced into *lacZ* also contain



A. NL250 B. NL350

Figure 7.2 The cell and nucleoid morphology of strains with *dif* located outside the replication terminus. Exponential phase cultures grown in LB broth at 37° were prepared for photography by condensation and staining of cell nucleoids with chloramphenicol and DAPI respectively (Materials and Methods). (**A**) NL250 (532 bp *dif* site at minute 8 of chromosome) (**B**) NL350 (532 bp *dif* site at minute 84 of chromosome). The long axis of each photograph represents approximately 70 μm. Condensation of the nucleoids does not interfere significantly with the phenotype demonstrated.

the *lacI*^{q} mutation, and therefore constitutively repress transcription of *lacZ*. Thus, it should be possible to control transcription through the *dif* sequence in these strains using IPTG (the *dif* sequence in these strains is the closest inserted sequence to the promoter). NL250 and NL350 were grown using LB media and broth with and without 100µg/ml IPTG. The phenotype of these cultures (untreated) was investigated, and a plasmid integration experiment carried out, calculating the integrated fraction of pLIM701 (pMAK705 *dif*$^{+}$)(see below and Chapter 4). In these experiments, results for NL250 and NL350 were not significantly different with and without IPTG (data not shown).

7.4 Verification of strain construction

The construction of strains with insertions in *lacZ* or in the *pst* genes was verified by analysis of their expression of beta-lacatamase and alkaline phosphatase respectively, and by Southern hybridisation.

The method of Miller, (1972), was used to assay beta-galactosidase activity in mutant strains grown on solid media. DS941 and derived strains produce a defective beta-galactosidase protein, as they contain a chromosomal *lacZΔM15* deletion (Vieira and Messing, 1982). Therefore, for beta-galactosidase activity through complementation, the alpha fragment of the protein must be provided (from eg. pUC18, Yanisch-Perron *et al.*, 1985). Therefore, DS941, and mutant strains with insertions in *lacZ* were transformed with pUC18, and plated on X-gal and IPTG. Colonies of DS941/pUC18 were dark blue, whereas all strains containing *lacZ*::532bp*dif*Gm^{R} or *lacZ*::Gm^{R} mutations were white, with the exception of NL232 and NL237, which were found to produce very pale blue colonies, as they also contain the *xerC*::*y17* mutation (Colloms *et al.*, 1990). This *xerC* allele contains a mini-Mu insertion into the *xerC* gene, and is a *lacZ* translational

fusion. Colloms *et al.*, (1990), report that this construct gave 1 to 5% of the fully derepressed level of expression from a chromosomal *lacZ* gene.

Assays for alkaline phosphatase expression by mutant strains followed the method of Wanner and Latterell, (1980). This uses the chromogenic substrate XP, similar to X-gal. XP (5-bromo-4-chloro-3-indoyl-phosphate-p-toluidine) is hydrolysed by phosphatases to give a blue pigment. Wild-type strains repress the expression of alkaline phosphatase (AP) when growing in media containing high levels of inorganic phosphate. Mutations in the *pst* genes interfere with this repression, causing constitutive expression of AP (Wanner and Latterell, 1980). Therefore, strains to be tested were plated on LB media containing XP (50 μg/ml) and a relatively high level of inorganic phosphate (2 mM). Whereas DS941 (wild-type) gave white colonies, all strains containing either *ΔpstCABphoU*::532bp*dif*Gm^R or *ΔpstCABphoU*::Gm^R mutations formed dark blue colonies.

Constructions were also verified by Southern hybridisation. The strains DS941 (wild-type), NL40 (*dif*), NL232, NL237, NL342 and NL347 were comparatively analysed. Genomic DNA from each strain was digested with *Pvu*II, separated on a 1% agarose gel and fixed to a synthetic membrane by capillary blotting. Sequential hybridisation with labelled DNA fragments from *dif*, *lacZ* and *phoU* gave the results shown in Fig. 7.3. Minor genomic bands when hybridising with *lacZ* and *phoU* probes were caused by incomplete stripping of the membrane before re-probing, and a contaminating fragment of pSN518 (the plasmid source of *pst* gene DNA) included in the *phoU* probe labelling mix. Probing with *lacZ* DNA not only shows hybridisation to the chromosomal *lacZ* gene, but to the mini-Mu element in the *xerC*::*y17* allele present in NL232, NL237, NL342 and NL347.

Using the previously described plasmid integration method (Chapter 4), the ectopic *dif* sites in strains NL250 (DS941 *difΔ6 lacZ*::*dif*) and NL350 (DS941 *difΔ6 pst*::*dif*) were shown to be active for site-specific recombination.

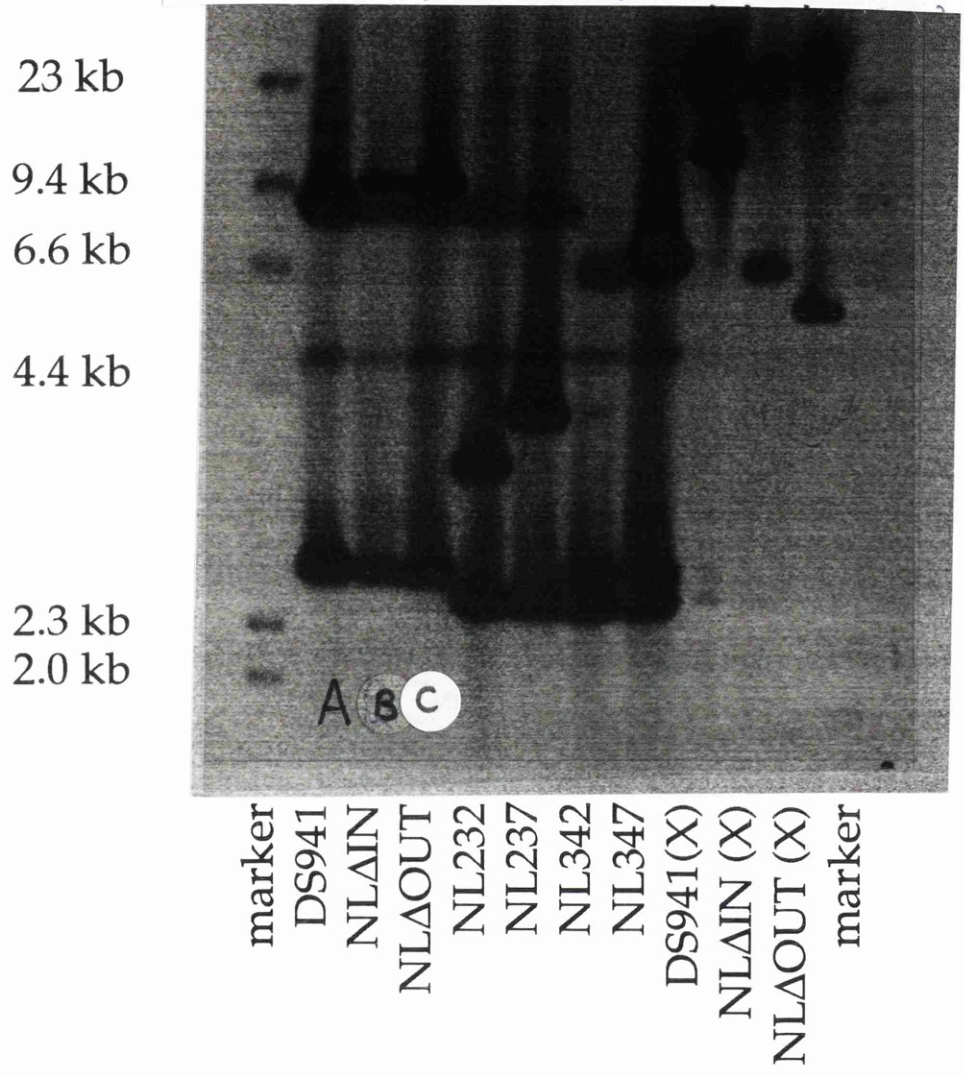
Figure 7.3. Strain verification by Southern hybridisation. Genomic DNA was prepared from each of the strains shown, and digested with a restriction enzyme, either *Pvu*II or *Xho*I (lanes containing DNA cut with *Xho*I are labelled (X) opposite, others are cut with *Pvu*II).

DS941	wild type
NLΔIN	*difΔ0IN*
NLΔOUT	*difΔ0OUT*
NL232	*difΔ6 lacZ*::Gm^R
NL237	*difΔ6 lacZ*::*dif*Gm^R
NL342	*difΔ6 pst*::Gm^R
NL347	*difΔ6 pst*::*dif*Gm^R

Samples were separated by 1.0% agarose gel electrophoresis and transferred onto a synthetic membrane by capillary blotting. A radiolabelled *Hin*dIII digest of bacteriophage lambda DNA was included as a marker. Hybridisation was performed using three different DNA fragments labelled by random primed strand synthesis, and gave the three seperate autoragiographs opposite,

A. A 532 bp *dif* fragment.
B. A ≈2.8 kb *Pvu*II *lacZ* fragment from p357.
C. A ≈1 kb *Mlu*I - *Hin*dIII *pst* fragment from pSN518.

Probes were removed with hot alkali, followed by neutralisation before re-probing.


23 kb
9.4 kb
6.6 kb
4.4 kb
2.3 kb
2.0 kb
A
B
C
marker
DS941
NLΔIN
NLΔOUT
NL232
NL237
NL342
NL347
DS941(X)
NLΔIN (X)
NLΔOUT (X)
marker

However, these strains gave a greatly reduced integrated fraction when compared to strains containing *dif* in the replication terminus region (Table 4.2). Possible reasons for this reduced integrated fraction are discussed below. Thus, ectopic *dif* sites, although active for site-specific recombination, appear to have no effect on the *dif/xer* cell morphology phenotype displayed by cells. In all of these experiments investigating the effect of the location of *dif*, strains retaining a *dif* site in the terminus region have wild-type cell morphology, whereas all strains lacking such a site displayed the charateristic filamentous *dif/xer* phenotype, regardless of other *dif* site sequences in the chromosome. Strains containing two copies of *dif* in different chromosomal locations were viable and produced colonies of approximately the same size as isogenic wild-type strains. It could of course be the case that ectopic *dif* sites are non-functional in these strains, as the plasmid integration method does not easily show that both recombination sites in the chromosome will take part in integration reactions.

7.5 Can the *loxP*/Cre recombination system functionally replace *dif* if inserted in chromosomal locations outside the replication terminus?

Activity of the *loxP*/Cre site-specific recombination system from bacteriophage P1 in the terminus region in place of *dif* is able to suppress the *dif/xer* cell morphology phenotype caused by deletion of the *dif* site (Chapter 5). In the experiments presented here, *dif* seemed unable to fulfil its function when in either of two other chromosomal locations. However, the reasons for this failure were unclear, and included the possibility that the functioning of *dif* sites is susceptible to interference by local factors (Chapter 4). Therefore, experiments were performed to investigate whether the *loxP*/Cre system

could suppress a *dif* deletion phenotype through activity in chromosomal locations outside the terminus region.

Mutant strains were constructed containing a *loxP* site introduced into *lacZ* at minute 8 or into the *pst* genes at minute 84 close to *oriC*, as had been previously done with *dif*. The same *loxP*Gm^R fragment from pN38 as had been introduced in previous *loxP* strains was inserted into the plasmids p357 and pSN518, sources of chromosomal DNA from *lacZ* and the *pst* genes respectively, to give pN81 and pN82 respectively. This produced two new mutations, *lacZ*::*loxP*Gm^R and *ΔpstCABphoU*::*loxP*Gm^R both analogous to previously constructed mutations containing 532bp *dif*$^+$ fragments. As *loxP* originates from bacteriophage P1, in order to avoid the use of P1 transduction, these two mutations were introduced into the chromosome by the method of Hamilton *et al.*, (1989), as discussed earlier (Chapter 5). These mutations were introduced into NL40, to give NL202 (NL40 *lacZ*::*loxP*Gm^R) and NL203 (NL40 *ΔpstCABphoU*::*loxP*Gm^R), analogous to the ectopic *dif* strains NL250 and NL350 respectively. The construction of these strains was verified by tests for the activity of beta-galactosidase and alkaline phosphatase, and by investigating Southern hybridisation of radio-labelled *loxP* oligonucleotides and a radio-labelled *dif*$^+$ restriction fragment to genomic DNA (Fig. 5.7, and data not shown). As described previously, the Cre recombinase, required for recombination at *loxP* was expressed from the plasmid pRH200 (Mack *et al.*, 1992). The strains NL202, NL203 and NL208 (*difΔ6*::*loxP*Gm^R) were transformed with pRH200 and pSD105 (an XerC expression plasmid) and their morphological phenotypes investigated by microscopic observation of untreated cultures. Although attempts were also made to demonstrate the recombinational activity of these chromosomal *loxP* sites in *lacZ* and the *pst* genes by plasmid integration of the temperature sensitive *loxP*$^+$ plasmid pN78 (Chapter 5, pMAK705 *loxP*$^+$), these were of limited success. The results

of these integration assays, and the phenotypes of strains tested is shown in Table 7.3.

This experiment fails to show any functional replacement of *dif* by the *loxP*/Cre recombination system located outside the terminus region. As with ectopic *dif* sites, plasmid integration experiments using these recombination sites give greatly reduced levels of integrants when compared to strains containing sites in the terminus region. Indeed, these experiments looking for plasmid integration into *loxP* sites in the *pst* genes and in *lacZ* give results that are not unquestionably above the basal levels achieved without active recombination.

7.6 Strains in which replication termination does not occur near the *dif* locus.

Neither *dif* nor *loxP* recombination appears able to suppress a *dif* deletion phenotype if outside the replication terminus region in these experiments. Why might this be the case? It could be that the positioning of *dif* in the terminus region is a prerequisite for its function in chromosome segregation. However, it could be that our experimental methods have interfered with this function through the local sequence context of ectopic sites. Results from experiments using *dif* in the terminus and results looking at *dif* and *loxP* dependent plasmid integration into ectopic sites might suggest that this was possible. How could the effects of chromosomal location be studied without effecting *dif* itself? Two approaches seemed obvious, either to move a much larger *dif* fragment to another chromosomal location (eg. 10 kb or bigger), or to change the location of the chromosomal area in which replication is completed, away from *dif*, by manipulation of the chromosomal terminator sites (*Ter* sites). The second method seemed 'cleaner' as only the

Strain and expression plasmid	Apparent phenotype	Integrated fraction with plasmid	
		pMAK705	pN78 (*loxP*)
NL202 + pSD105	filamentous	nd	nd
NL202 + pRH200	filamentous	nd	6.0×10^{-4}*
NL203 + pSD105	filamentous	nd	1.1×10^{-5}
NL203 + pRH200	filamentous	2.1×10^{-5}	6.4×10^{-4}*
NL208	filamentous	4.0×10^{-4}	6.7×10^{-5}
NL208 + pRH200	wild-type	9.0×10^{-4}	$1{,}0 \times 10^{-2}$

Table 7.3. Plasmid integration assays looking for *loxP*/Cre dependent integration into different chromosomal locations The calculation of integrated fractions is described in Chapter 4. Results marked '*' were calculated as the mean value from two independent experiments. The results using NL208 were calculated as the mean value from several independent experiments and are also contained in Table 4.2. 'nd' represents values not determined. NL208, NL202 and NL203 contain *loxP* sites introduced into a deletion of the *dif* region, the *lacZ* gene and the *pst* genes respectively. pSD105 and pRH200 express XerC and Cre respectively.

point of replication termination should be changed, *dif* itself should remain unaffected.

dif is naturally located approximately 16 kb from *TerC* at one end of the region between the innermost terminator sites *TerC* and *TerA*, approximately 250 kb long, in which the termination of replication normally occurs (Fig. 7.1; Bouché *et al.*, 1982; de Massy *et al.*, 1987; Hill *et al.*, 1987). All *Ter* sites characterised consist of a short (23 bp) DNA sequence that when bound by the Tus protein, acts as a unidirectional block to the progression of replication forks along the DNA (Hidaka *et al.*, 1988; Hill *et al.*, 1988, 1989). These sequences share a common concensus with terminators from other circular genomes in prokaryotes, plasmids RK6, R100 and others, and the *Bacillus subtillis* chromosome (Bastia *et al.*, 1981; Carrigan *et al.*, 1987; Hidaka *et al.*, 1988; Hill *et al.*, 1988). By introducing a terminator site mid-way between *TerA* and *TerC* in the same orientation as *TerC*, replication forks should be unable to progress through to *dif* and *TerC*, termination occuring in the chromosomal region between *TerA* and this new terminator. This is demonstrated in Fig. 7.4. Therefore, it was decided to introduce a novel chromosomal replication terminator site into DNA close to the *trg* gene, approximately 90 kb away from *dif* (Fig. 7.4). This should ensure that the two chromosomal replication forks meet at least 90 kb away from *dif*. Would this interfere with the functioning of an otherwise wild-type *dif* site?

A new *Ter* site was introduced into the chromosome using the same strategy used and described previously for the introduction of recombination sites (Chapter 4, Chapter 5). The plasmid pPM1000 was used as a source of DNA close to the *trg* gene (Moir *et al.*, 1992). As this plasmid is approximately 28 kb long, a *Pst*I restriction fragment (≈8kb) was cloned into pUC8 to give pN85, containing unique *Kpn*I and *Eco*RV restriction enzyme sites with substantial chromosomal flanking sequences on both sides. This would allow the introduction of a *Ter* site at minute 31.6 of the chromosomal genetic

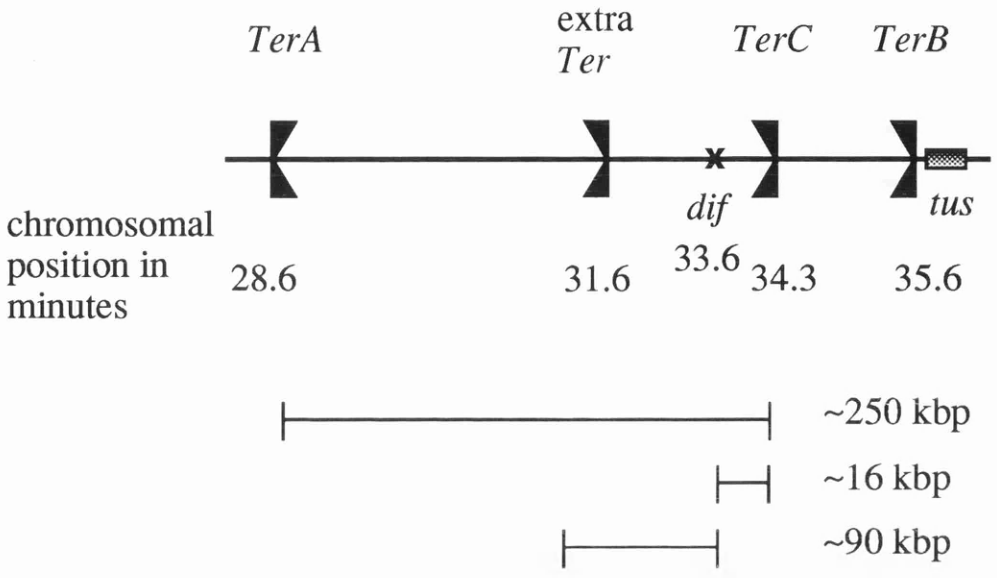


Figure 7.4. Insertion of an extra *Ter* site into the replication terminus region. This representation of the *E. coli* replication terminus region shows the position into which an extra *Ter* site was introduced experimentally. Three of the six identified natural chromosomal *Ter* sites are shown, represented such that replication forks approaching from the flat side are not effected (eg. *TerC* halts replication forks travelling from left to right). Chromosomal locations in minutes refer to the genetic linkage map of Bachmann, (1990). Some distances are also shown in kilobases, estimated from the chromosomal physical map of Kohara *et al.*, (1987).

linkage map (Bachmann, 1990), kilobase 1520 of the physical map (Kohara *et al.*, 1987).

The *TerB* sequence, a chromosomal *Ter* site, also known to be sufficient to halt replication forks in a plasmid environment (Hill *et al.*, 1988), was provided as two complementary synthetic oligonucleotides containing this sequence and a *Sca*I site (Table 2.3). These were annealed, producing DNA ends compatible for ligation into, and reformation of, *Asp*718 and *Sst*I sites, and ligated into pUC18 and pUC19 to give pN90 and pN91 respectively. In order that plasmids containing replication terminator sequences could be constructed and used in the production of mutant strains, plasmids were maintained in, and prepared from, the *tus* strains TH210 (PK2619, Hill *et al.*, 1989 a gift of Tom Hill). For the selection of mutants during construction, a *Ter* site/gentamicin resistance gene plasmid was produced by cloning the *Ter* site from pN90 into the polylinker of the Gm^R plasmid pN10, giving pN93, and this *Ter* site/Gm^R fragment cloned into the chromosomal DNA of pN85 to give pN94. This mutation was then introduced into the chromosome of JC7623 (*recBCsbcBC*) by linear transformation, and then transduced into both AB1157 and DS941 (*recF*), giving NL100 and NL102 respectively.

The two *Ter* site containing plasmids pN90 and pN91 are identical apart from the orientation of their polylinkers and inserted *Ter* sequences. The *Ter* site in pN91 should arrest replication forks initiated at the plasmid's uni-directional ColE1 origin, whereas the site in pN90 should have no effect. Differences between the transformation efficiency of tus^+ strains with DNA of these two plasmids prepared from tus^- strains, differences in transformant colony size, and the preparation of plasmid DNA from tus^+ strains strongly suggest that the artificially constructed *Ter* sequence present is able to arrest the progress of replication forks when in the correct orientation in these plasmids.

Microscopic investigation of the cell morphology of untreated exponentially growing cultures of NL100 (AB1157::*extra Ter*) and NL102 (DS941 ::*extra Ter*) did not detect any differences between them and wild-type strains (data not shown). Possible reasons for this are discussed below.

7.7 Discussion

Neither the *dif*/Xer nor the *loxP*/Cre recombination system was able to suppress the phenotype caused by deletion of the chromosomal *dif* site, when inserted in the chromosome in the *pst* genes or the *lacZ* gene. This lack of functional replacement could have several explanations. It might be expected that in order to allow normal chromosome segregation, *dif* must function in the replication terminus region, or perhaps even in a specific location within the replication terminus. This lack of suppression by sites outside the terminus region could be caused by the physical separation of the two copies of a site after replication. Once copied, non-terminus sequences might be expected to be rapidly physically separated by an active partition mechanism acting upon the replicating chromosome. This separation would prevent recombination between two ectopic *dif* copies on sister chromosomes. Sequences in the terminus region may not only be the last to be replicated, but may remain in physical proximity after replication for longer periods than sequences located elsewhere, thus maximising their recombination potential. If the function of *dif* recombination is the resolution of chromosome dimers, only after chromosome replication is completed would the dimeric or monomeric form of the chromosome copies become evident. However, recombination could only occur between two copies of a resolution site if they were still in physical proximity.

It is also possible that recombination sites inserted outside the terminus region do not functionally replace *dif* because recombination at these sites does not occur with the required efficiency, or that they are otherwise limited by their local environment (as discussed in Chapter 4). Results of plasmid integration experiments might support this, as the integrated fraction using *dif* or *loxP* containing plasmids was approximately 50 to 100-fold lower in strains in which the correct chromosomal recombination site was located outside the terminus region, as compared to strains containing these sites in the wild-type location of *dif*. However, as described elsewhere (Chapter 4), these experiments may not give a direct indication of the rate of integrative recombination. Other possible explanations for this reduced integrated fraction have been considered. Because terminus sequences are copied last in each cell cycle, integration of a plasmid into the terminus should almost always lead to both daughter cells having a plasmid copy integrated, and might lead to a higher integrated fraction in experiments. However, such a difference would be expected to be very small. It was thought that the replication termination system, by halting replication forks exiting the terminus, could minimise the detremental effects to cell growth of inserting an active plasmid replication origin into the chromosome (Yamaguchi and Tomizawa, 1980) if this inserted origin were within the terminus region, and might cause a great measured over-representation of integration events into the terminus relative to other locations. However, since plasmid integration with pLIM701 (temperature sensitive *dif*$^+$ plasmid) into the *tus* strain NL105 (DS941 *tus*) gives a similar result as other *dif*$^+$ strains, this seems not to be the case.

The insertion of an extra *TerB* sequence into the chromosome mid-way between *TerC* and *TerA*, oriented to halt replication forks travelling towards *TerC* and *dif*, appeared to have no detectable effect on cell morphology. As this experiment was very much unfinished, it is difficult to draw conclusions

from this result. Although the artificially produced terminator sequence appears to be functional for the arrest of replication in the context of some plasmids, whether this is the case in the construct introduced into the chromosome, either on a plasmid, or in the chromosome, has not been determined. Indeed, results were published while these experiments were being performed, that show that the arrest of replication forks by *E. coli* terminator sequences, at least in plasmids, can be very much dependant upon the sequence context of the *Ter* sequence used (Bierne *et al.*, 1994). Thus, the lack of a phenotype caused by the introduction of a *TerB* sequence in the experiments described here could mean that the arrest of chromosomal replication 90 kb away from *dif* has no effect on cell morphology. However, it could simply mean that local factors effecting the function of the inserted replication terminator sequence or other experimental problems mean that the progression of replication forks towards *dif* is not halted in NL100 and NL102.

Chapter 8

Concluding remarks

Concluding remarks

Is it site-specific recombination or some other process that occurs at *dif* to ensure normal *E.coli* chromosomal segregation and cell division? Here, three pieces of data are presented that strongly point to it being site-specific recombination. Firstly, a 33bp fragment containing the 28bp *dif* core site, that is functional in site-specific recombination *in vivo* plasmid assays, is sufficient for normal chromosomal segregation and cell division when placed in either orientation in its normal position in the replication terminus region. Secondly, mutations in the putative active sites of XerC or XerD interfere with both site-specific recombination and chromosome segregation/cell division. Lastly, Cre-mediated site-specific recombination at a *loxP* site inserted into the replication terminus region can functionally replace Xer recombination at *dif*, as judged by suppression of the aberrant cell nucleoid morphology of a *dif* deletion. Since *loxP* has little sequence similarity to *dif*, and because suppression requires Cre, it seem most reasonable that the process of site-specific recombination leads to normal chromosome segregation.

It has been previously proposed (Blakely *et al.*, 1991; Kuempel *et al.*, 1991; Sherratt *et al.*, 1993) that the function of Xer site-specific recombination is to 'undo the damage' of relatively rare homologous recombination events: odd numbers of exchanges between circular chromosomes will generate dimeric molecules that cannot be segregated effectively. If so, the viability and substantial proportion of normal cells in *dif*$^-$ or Xer$^-$ populations seems to indicate that many cells do not encounter a problem in segregation and cell division, presumably because an odd number of homologous exchanges occurs relatively rarely, perhaps at most every few cell generations (Forro and Wertheimer, 1960; Kuempel *et al.*, 1991). Xer recombination at natural plasmid sites (e.g. *cer* in ColE1) is exclusively intramolecular, and is therefore ideally suited to convert multimers to monomers. This resolution selectivity requires about 190 bp of accessory sequences adjacent to the core recombination site

and accessory proteins (Stirling *et al.*, 1988b; Stirling *et al.*, 1989; Summers, 1989; Blakely *et al.*, 1993). It seems probable (Blakely *et al.*, 1993; Sherratt, 1993; S. Colloms unpublished) that the mechanism by which this selectivity is enforced is similar to that used by the Tn3 *res*/resolvase and related systems, which show a strong selectivity for either resolution (e.g.. *res*/resolvase) or inversion (e.g.. *gix*/Gin). In all of these cases, accessory proteins and accessory sequences appear to be involved in the formation of a recombinational synapse which has the complexity to direct the DNA into a precise synapse topology (e.g. see Stark *et al.*, 1989a; Stark and Boocock, 1995). We have no evidence that recombination at *dif* can show resolution selectivity; recombination in plasmid substrates occurs inter- and intra-molecularly and no data support the involvement of accessory sequences outside of the *dif* core site or additional accessory proteins. How then does recombination at *dif* convert putative chromosomal dimers to monomers? Either there is selectivity for intramolecular resolution by an unknown mechanism that does not operate when *dif* is present in multicopy plasmid and does not require accessory sequences, or recombination at *dif* in the terminus region of the chromosome leads to chromosome segregation despite the lack of resolution selectivity.

Two ways in which unconstrained recombination at *dif* could lead to effective chromosome segregation have been proposed (Fig. 1.9; Blakely *et al.*, 1991; Kuempel *et al.*, 1991; Sherratt *et al.*, 1993). In one, rapid recombination between newly replicated *dif* sites would ensure that chromosomes were monomeric 50% of the time, irrespective of whether homologous recombination had acted; they could therefore be physically separated by a chromosome partition mechanism. The second model relies on Xer recombination proceeding by a Holliday junction intermediate, as would be expected for this class of enzyme (Stark *et al.*, 1992). Holliday junction formation by site-specific recombination at two chromosomal *dif* sites would

produce 'figure-eight' molecules irrespective of whether or not there had been a homologous recombinational exchange. If the process that leads to chromosome segregation now begins to separate the daughter chromosomes linked by the Holliday junction, a resolution event that senses the local conformational changes induced by separation could lead to monomeric daughter chromosomes. There is no evidence to support this latter hypothesis other than the *in vivo* accumulation of Xer-mediated Holliday junction-containing molecules under some conditions (McCulloch *et al.*, 1994). Neither of these two possible modes of function can be excluded, although the experiments presented here, particularly the functional replacement of *dif* by the *loxP*/Cre recombination system, would seem to favour the first, simpler model.

Recombination between copies of any hypothetical circular replicon will produce dimeric forms. Thus, any replicon for which multimerisation might pose a threat and that is susceptible to significant levels of recombination between replicated or replicating copies of its sequence, should require a mechanism for multimer resolution, or possibly multimer avoidance. It has been suggested that high levels of homologous recombination could fulfil this role, particularly for high copy-number replicons (or possibly a low concentration of their DNA), for which multimeric forms pose a less clear threat (e.g.. possibly some plasmids or organelle genomes). However, it might be suggested that most circular bacterial genomes require a dedicated resolution system. Consistent with this hypothesis, multimer resolution systems have been identified in many natural plasmids, and importantly, evidence to support the widespread existence of systems homologous to the *E. coli dif*/Xer system in other bacterial chromosomes is developing. Genes homologous to *xerC* and *xerD* are known to be widely distributed amongst the bacteria (G. Blakely, unpublished work), although few have been cloned and characterised. A

notable exception is the *Pseudomonas aeruginosa SSS* gene, an *xerC* homologue, sharing 49.8% corresponding amino acid sequence identity, that can complement an *xerC* mutant in Xer plasmid recombination assays (Höfte *et al.*, in press). Since *SSS* mutants show a somewhat filamentous phenotype, this suggests a possible conserved function in chromosome segregation. Work is currently ongoing, attempting to characterise Xer-like systems from *Bacillus subtilis* and *Salmonella typhimurium* and to identify *xerD* and *dif* like sequences from *P. aeruginosa*. If this work is successful, it may be very informative about the functioning of *dif*.

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