COLE1, *CER* SITE-SPECIFIC RECOMBINATION

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Dedicated to Ged

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Abbreviations

(i) Chemicals

Ac - acetate

APS - ammonium persulpate

ATP - adenosine triphosphate

BSA - bovine serum albumin

DNA - deoxyribonucleic acid

d(NTP) - 2'-deoxy (nucleotide)

DTT - dithiothreitol

EDTA - ethylene diamine tetra-acetic acid (disodium salt)

EtBr - ethidium bromide

EtOH - ethanol

FSB - final sample buffer

SCFSB - single colony final sample buffer

IPTG - isopropyl B-D thiogalactopyranoside

RNA - ribonucleic acid

RNase - ribonuclease A

SDS - sodium dodecyl sulphate

TEMED - NNN' N' tetramethyl ethylenediamine

Tris - tris (hydroxymethyl) amino ethane

X-gal - 5-bromo, 4-chloro, 3-indolyl, B-D galactoside

(ii) Antibiotics

Ap - ampicillin

Cm - chloramphenicol

Kan - kanamycin

Nal - naladixic acid

Rif - rifampicin

Str - streptomycin

Tc - tetracycline

(iii) Phenotype

X^r - resistance to X

X^s - sensitivity to X

oriV - replication origin

1^m - monomer

2^m - dimer

(iv) Measurements

mA - milliamps (10^{-3} A)

bp - base pair

kb - kilobase pair (10^3 bp)

kd - kilodalton (10^3 dalton)

^oC - degrees centigrade

Ci - curie

uCi - microcurie (10^{-6} curie)

g - centrifugal force equal to gravitational acceleration

g - gramme

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

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

l - litre

ml - millilitre

ul - microlitre

M - Molar

mM - millimolar

uM - micromolar

m - metre

cm - centimetre (10^{-2}m)

mm - millimetre (10^{-3}m)

mins - minutes

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

sec - seconds

v - volts

w - watts

(v) Miscellaneous

ln - natural log

CAT - chloramphenicol acetyl transferase

UV - ultra violet light

Tn - transposon

pfu - plaque forming units

moi - multiplicity of infection

aa - amino acids

rt - room temperature

% - percentage

LMP - low melting point

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SUMMARY

The multicopy, naturally occurring plasmid ColE1 is maintained stably under normal growth conditions, whereas plasmid cloning vectors related to it are relatively unstable. Summers and Sherratt (1984) presented evidence suggesting that ColE1 is partitioned randomly at cell division and that plasmid stability is inversely correlated with plasmid multimerization. Wild type ColE1 is stable as it utilizes a site-specific recombination system to breakdown multimers formed by homologous recombination. This increases the stability of ColE1 by maximising the number of independently segregating units.

The ColE1 site-specific recombination system utilizes a site *cer*, contained in a 238bp fragment (3731-3969bp) and a recombinase *xer*, which is probably host encoded. To investigate if the recombinase acting at *cer* was host encoded, mutants affecting *cer*-specific recombination were sought. A total of ten Tn5::*xer* mutants have been isolated, only three of which have been studied in any detail. These three host mutants are called DSX.1, DSX.2 and DSX.300. DSX.2 and probably DSX.1 are spontaneous mutants, whereas DSX.330 is a Tn5::*xer* mutant.

These *xer* mutants have been used to demonstate: (i) a correlation between multimerization and instability for ColE1, (ii) that ColE1 cannot supply in *trans* any of the functions absent in the *xer* mutants and (iii) that the *xer* functions are used in the multimer resolution systems of the high copy number plasmids ColK, CloDF13, and perhaps ColE2 and ColE3.

The *xerA* gene mutant in DSX.330 has been cloned and sequenced. Sequence analysis of the minimum complementing clone reveals an ORF, which would encode for a polypeptide of 17kd, whose presence has been confirmed using whole cell protein extracts and minicells. The *xerA* plasmid clone complements the Xer$^-$ phenotype of DSX.300 but not that of DSX.1 and 2, defining at least two

host complementation groups necessary for cer-specific recombination.

Classical genetic techniques were then used to determine the genetic map position of the xer genes. The data indicates that xerA mutant in DSX.300 lies between 82 and 87mins, whereas the xer1 and 2 gene(s) mutant in DSX.1 and 2 lie between 87 and 91mins. One of these xer genes is probably the recombinase, whereas the other may be a DNA binding protein, involved in the formation of higher order protein-DNA structures or in DNA bending.

CHAPTER 1

INTRODUCTION

1.1 General Introduction

Plasmids and phages were probably derived from host chromosomes by excision of a fragment of DNA, including a site for initiating autonomous replication. This cyclic DNA could then have evolved by gene duplication, mutation and incorporation of additional host DNA. These replicons evoloved structures for their transfer, plasmids developing pili for conjugation and phages a means of infection. By these mechanisms phages developed the ability to spread without cell to cell contact at the expense of their hosts; whereas plasmids have developed more symbiotic relationships with their hosts, increasing their chances of survival by gaining genes useful to the host.

Plasmids are on the whole double stranded covalently closed DNA molecules, however a number of exceptions ex ist e.g. the linear plasmid pSLA2 in Streptomyces rochei (Hirochika et al, 1984). Plasmids have been isolated from a spectrum of bacterial species including gram-positive, gram-negative and Archaebacteria (Broda, 1979). Additionally plasmids have been found in lower eukaryotes, the most noteable example being the 2 micron circle from Saccharomyces cerevisiae (Broach, 1982). Plasmids encode for a variety of phenotypic traits including determinants for drug resistance, resistance to metal ions, production of bacteriocins ,production of enterotoxins and altered sensitivity to mutagens (Broda, 1979). Additionally plasmids encode for determinants responsible for control of initiation of vegetative replication, for their efficient partition at cell division and for the means to transfer horizontally between cells in a population.

Natural plasmids can vary in size from a few kilobases to a few hundred kilobases and their copy number per cell can vary from one to hundreds. In general it would be true to say that large plasmids are normally present at low copy number and small

plasmids are present at higher copy numbers. Plasmids control initiation of their replication, which in turn controls the copy number of that plasmid. The host is responsible for providing most of the gene products necessary for plasmid DNA replication (Scott, 1984). The plasmid encoded replication functions are confined to a small region of the plasmid, consisting of a replication origin (ori), a replication initiator (rep) and replication control functions (cop).

1.2 Stable inheritance of plasmids.

There are two conditions which must be fulfilled in order that a plasmid can be stably maintained at a constant steady state copy number in a population. The first of these is that the rate of plasmid replication must be actively controlled by a system capable of assessing copy number and able to correct any deviation from the average copy number (Nordstrom et al, 1984). The second condition, which must be fulfilled is that a plasmid must have an efficient means of partition to daughter cells at cell division. These two parameters are now examined in more detail.

1.2.1 Control of initiation of replication

The copy number of any plasmid is a function of the plasmid, the host and the growth conditions. The replication control mechanism of any plasmid determines the average number of replications per cell per cell cycle. It can be assumed that control of replication is exerted at the level of initiation of replication and not elongation (Pritchard, 1969, 1974), as replication of even a large plasmid takes only 1 to 3 minutes. Nordstrom et al (1972), have presented evidence showing that plasmids control their own replication rates. This was shown

using copy number mutants of R1, which have a higher steady state copy number than their wild type parent. These copy number mutants can be transfered from one cell to another by conjugation or transformation and still maintain higher copy numbers. This implies that the mutations affecting plasmid copy number are plasmid encoded.

There are also host mutations affecting the copy number of certain plasmids, such as F (Cress and Kline, 1976) and R1 (Nordstrom et al,1972). Most of these are uncharacterised but for example mutants in the RNA polymerase gene would affect plasmid copy numbers as they would specifically affect expression of plasmid promoters necessary for plasmid replication.

There are two ways in which a plasmid may control initiation of replication either by a "passive" replication control system as defined by Pritchard (1978), or by an active control system using a plasmid-specific negative-feedback loop as proposed by Pritchard et al, (1969) and Sompayrac and Maale, (1973).

An example of a passively controlled system is one in which the rate of plasmid replication is determined by a "constitutive" origin of replication, which is only able to bind the replication initiation complex once per cell cycle on average. If the average frequency of replication was exactly once per cell cycle , the statistical distribution around this average would cause an extensive drift in plasmid copy number in the population. In this sort of system the variations in the plasmid copy number would be inherited as this system is unable to correct deviations from the normal, hence a "passive" replication control system seems unlikely.

In an active control system deviations in the plasmid copy number are detected and the frequency of initiation of replication adjusted accordingly. To do this a replicon-specific negative feed-back loop is required to correct any deviations from the average copy number. An example of such a system is a plasmid

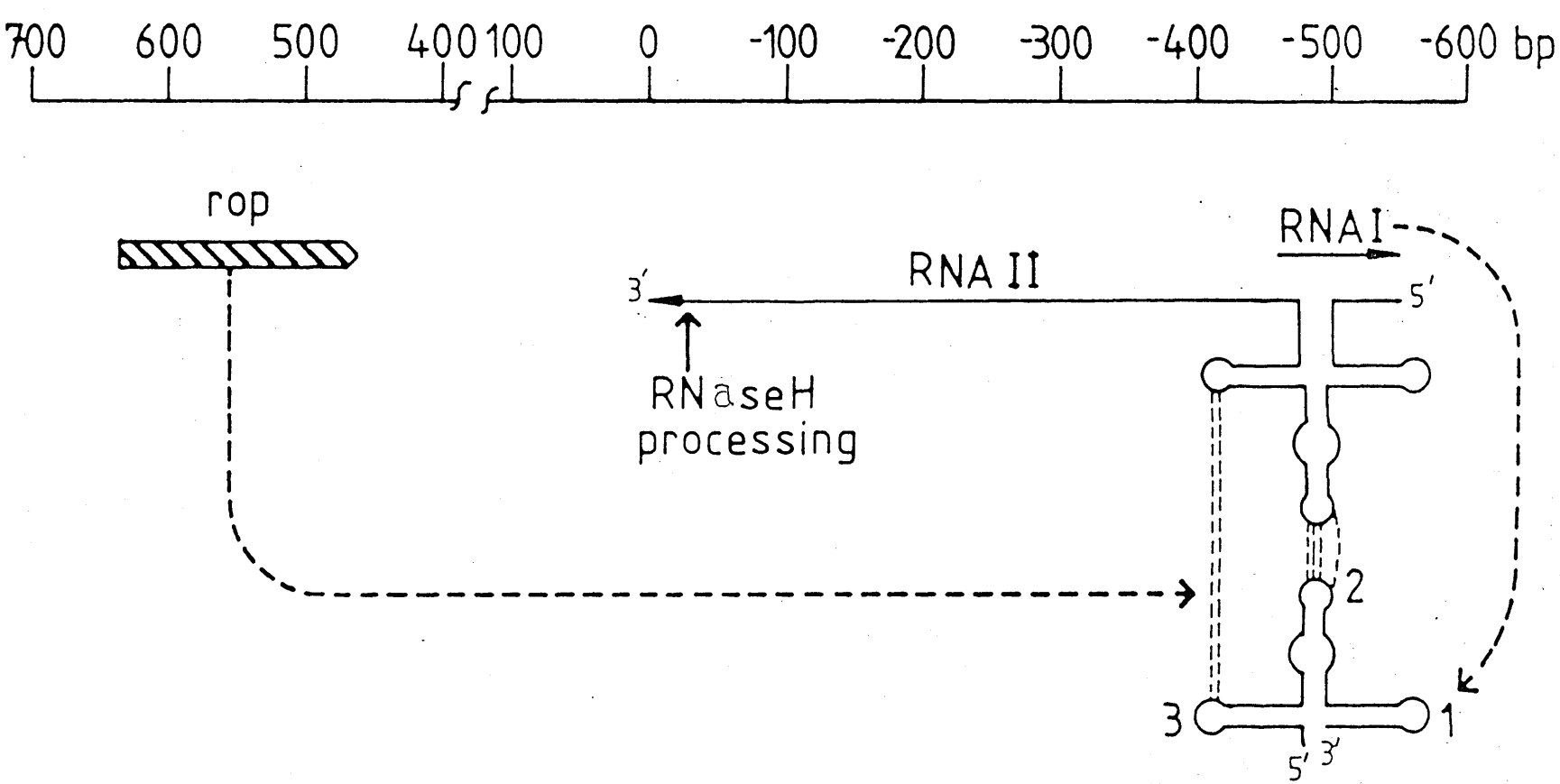

Figure 1.1 The replication control region of ColE1. Interaction between the complementary RNA I and RNA II molecules prevents primer processing by RNaseH to form the active replication primer. The Rom protein catalyses the interaction between RNA I and RNA II, thereby enhancing the negative control mechanism (Scott, 1984).

encoded inhibitor, which controls the rate of expression of a primer necessary for plasmid DNA replication. These ideas have been developed by Pritchard (1978) and Pritchard and Grover (1981). In this type of system if the plasmid copy number increases above the average, then the inhibitor concentration rises, which causes a decrease in the average rate of initiation of plasmid replication. The copy number of the plasmid is affected by parameters such as the synthesis of the inhibitor (constitutive or subject to autoregulation) and the rate of interaction of the inhibitor with the target. If any of these parameters are altered then the copy number of the plasmid will be affected.

Three types of plasmid replication inhibitors have been demonstrated in different plasmids: (i) Small RNA molecules e.g. RNA I of ColE1 (Tomizawa et al, 1981); and copA of R1 (Stougaard et al, 1981) (ii) Small proteins e.g. the ColE1 rom (rop) encoded protein (Tomizawa and Som, 1984) and R1 encoded copB (Molin et al, 1981) (iii) A series of direct repeats of DNA e.g. F (Tolun and Helinski, 1981) and P1 (Chattoraj et al, 1984).

The best way to illustate a replicon-specific negative feed-back loop is to look at a specific plasmid replication system. The plasmid system chosen was ColE1 and there were two reasons for the choice: (i) replication control of ColE1 has been extensively studied and (ii) ColE1 was the plasmid studied in this thesis. Control of ColE1 replication involves two plasmid encoded factors, which negatively control plasmid copy number by inhibiting initiation of replication of ColE1 (Figure 1.1). The first of these negative regulators is a small RNA molecule, called RNAI. This molecule can interact with the unprocessed RNA primer of ColE1 replication (RNAII) and prevent primer processing by RNase H (Tamm and Polisky, 1983). Thus RNAI acts as a negative regulator of initiation of replication by limiting the amount of effective primer available. The same RNAI-RNAII

interaction is responsible for the incompatibility properties of ColE1 (Tomizawa and Itoh, 1981). The second negative regulator is a 63 amino acid polypeptide, which is the product of the rom gene. The Rom polypeptide acts to enhance the binding of RNAI to RNAII and therefore enhances the inhibitor activity of RNAI on primer formation (Tomizawa and Som, 1984). Mutants of the rom gene have increased copy numbers (Twigg and Sherratt, 1980), suggesting that rom may be involved in the modulation of plasmid replication by limiting the amount of primer precursor available for RNase H processing.

ColE1 uses two replication inhibitors an RNA and a small protein. The average copy number of ColE1 is 20 copies per cell at cell division (Timmis, 1981). If the copy number in any cell increases above the average then there is an increase in inhibitor and hence a decrease in initiation of replication and vice versa, maintaining an average copy number per genome equivalent.

1.2.2 Partitioning of plasmids at cell division.

Most naturally occurring plasmids are rarely lost from cells. This implies that a plasmid has an active plasmid copy number control system and an effective means of partitioning plasmids to daughter cells at cell division. There are two ways in which plasmids may behave at cell division. They can be distributed randomly at cell division to daughter cells or by an active mehanism. If a plasmid is partitioned by an active mechanism then it should be stable, whereas a random partitioning mechanism will result in the production of plasmid frees at a frequency related to the copy number of the plasmid in the dividing cell.

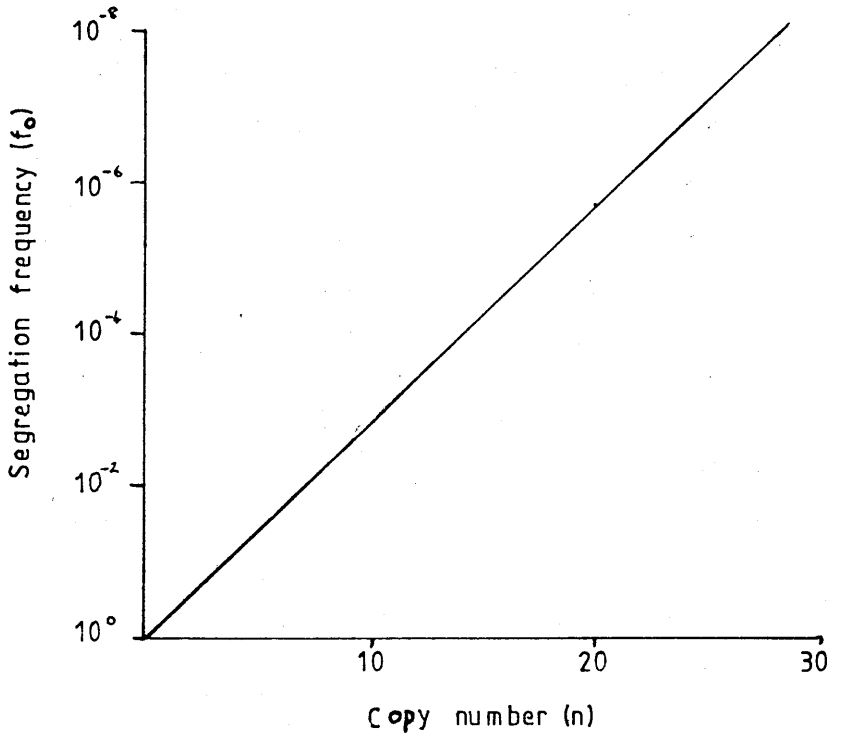

Figure 1.2 Relationship between segregation frequency and plasmid copy number. The relationship is derived from the binomial distribution and may be expressed mathematically as: $f_o = 2^{1-n}$, where f_o is the frequency of plasmid free cells per generation and n is the copy number of the plasmid in the dividing cell (Summers and Sherratt, 1985).

(A) Partitioning of low copy number plasmids.

The relationship between segregation frequency and copy number (Figure 1.2) was proposed by Summers and Sherratt (1984) and is derived from the binomial distribution. This relationship can be represented thus, $f_o = 2^{1-n}$, where f_o is the frequency of plasmid free cells per cell per generation and n is the copy number of a plasmid at cell division. If a plasmid has a low copy number and is distributed randomly to daughter cells, plasmid free cells would be generated very frequently leading to the rapid loss of the plasmid from the cell population. The experimental data available for low copy number plasmids: pSC101 (Meacock and Cohen, 1980), R1 (Nordstrom et al, 1980) and F (Ogura and Hiraga, 1983) indicate that strains containing these plasmids segregate plasmid frees very rarely. Therefore an active partitioning mechanism must operate in these low copy number plasmids to ensure that each daughter cell receives a copy of the plasmid at cell division.

Deletion analysis of low copy number plasmids indicates that a region of the plasmid is essential for its stable maintenance. This region is called par or stb and has been shown to be essential for the plasmids NR1 (Miki et al,1980), R1 (Nordstrom et al, 1980), F (Ogura and Hiraga, 1983) and pSC101 (Meacock and Cohen, 1980). The par region of pSC101 has been localised to a 370bp fragment, adjacent to the origin of replication. Deletion of par results in decreased stability, which is in agreement with the random distribution of plasmid copies to daughter cells at cell division (Nordstrom et al, 1980). par acts in the cis but not in the trans configuration and has been shown to be functionally and physically separable from the replication controlling regions of the plasmid. The par region of pSC101 also stabilizes other unrelated plasmids, such as pACYC184, a p15A derivative. Filter binding experiments have shown that the

par region of R1 and pSC101 is associated with the outer membrane of bacterial cells (Gustafsson et al, 1983), suggesting that these plasmids may be membrane associated at cell division.

The region of F required for its stable maintenance has been localized to a 3kb region, adjacent to the replication region. Analysis of this region has revealed two loci, sopA and sopB which, specify trans acting functions, and a third locus sopC, that is cis-acting (Ogura and Hiraga, 1983). The model proposed by Ogura and Hiraga (1983) for the partition of F involves a specific interaction of the cis acting sopC region of the plasmid with the plasmid encoded sopB protein and at least two host encoded proteins of 75 and 33kd (Hayakawa et al., 1985). sopC is thought to bind to the cellular membrane via the sopB protein and the two chromosomally encoded proteins. The role of the sopA protein is some what obscure, since it does not seem to bind to sopC, however, it may be involved in regulating expression of the sopB gene (Ogura and Hiraga, 1983). Plasmids carrying the sopC and sopB loci were incompatible with plasmids carrying the same segment of F. This has been interpreted as implying that there is competition between sopC regions for a limited number of binding sites in the membrane. This phenomenon of incompatibility has also been noted for par$^+$ R1 plasmids (Nordstrom et al, 1981).The par region of pSC101 requires no diffusable gene products and does not show incompatibility with another par$^+$ pSC101 (Gustafsson et al, 1983).

It has been shown that cell division is inhibited if replication of the low copy number plasmid, F is inhibited (Miki et al, 1984), in the same way as cell division is inhibited if chromosomal replication is incomplete.

In some low copy number systems efficient partitioning may not be possible if multimers are formed, as these reduce the number of independently segregating plasmid units. The phage P1 behaves as a low copy number autonomously replicating plasmid in the

prophage state. To deal with the formation of multimers by homologous recombination P1 encodes a site-specific recombination system to convert oligomers to monomers. This system utilizes a site loxP on P1 and a recombinase encoded by the phage gene, cre (Austin et al, 1981; Abremski et al, 1983).

(B) Partitioning of high copy number plasmids.

A variety of models have been proposed for the partitioning of high copy number plasmids, including purely random models (Durkacz and Sherratt, 1973), active partitioning models (Della Latta et al, 1978) and models involving both active and random partition (Hashimoto-Gotoh and Ishii, 1982). To date there have been no convincing reports of an active partitioning mechanism for high copy number plasmids. The evidence available points to a random mechanism of partition of high copy number plasmids at cell division. If multicopy plasmids like ColE1, ColK and CloDF13 are partitioned randomly then the probability (f_o) of generating a plasmid free cell is given by the binomial distribution. Thus the relationship is $f_o = 2^{(1-n)}$, where n is the number of plasmid copies prior to cell division. If ColE1 has a copy number of approximately 20 (Timmis, 1981) and is partitioned randomly, then plasmid frees would not be detectable i.e. $<10^{-5}$. This is true for ColE1, however many of the cloning vectors related to ColE1 have copy numbers greater than 20 and yet are highly unstable. How can one account for this anomaly? The instability of plasmid cloning vectors in comparison to the natural plasmids can be accounted for by host homologous recombination, generating multimers. Summers and Sherratt (1984), proposed that a major cause of increased plasmid copy number variance and hence plasmid instability was the generation of multimers by homologous recombination. An origin counting hypothesis (Summers and Sherratt, 1984; Jones, 1985), predicts

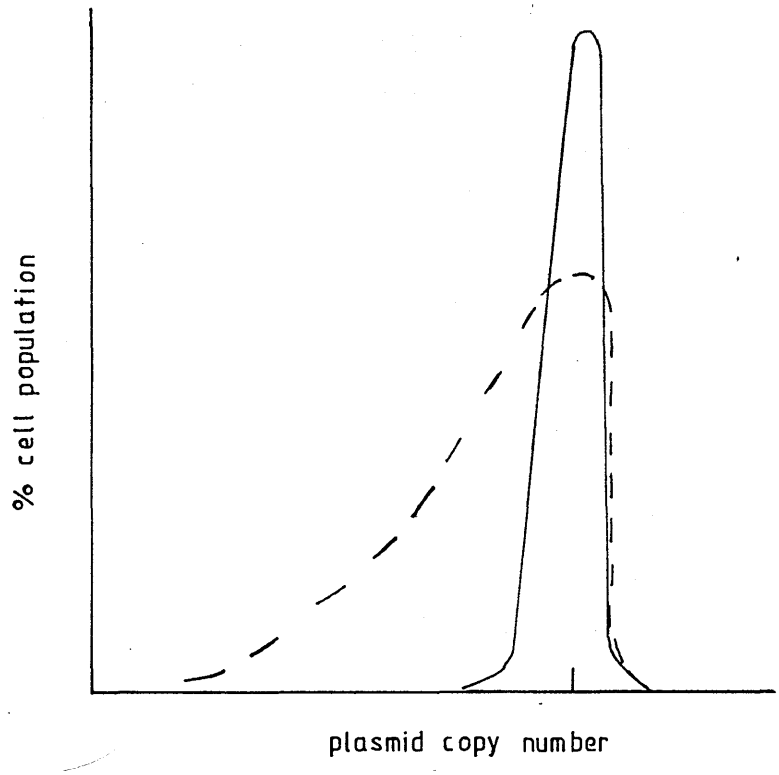

Figure 1.3 The predicted effect of inter-plasmidic homologous recombination in the distribution of plasmid copy number.

(- -) represents the distribution of copy number immediately before division in a population of bacteria proficient in homologous plasmid recombination.

(—) represents the distribution of copy number immediately before division in a population of bacteria deficient in homologous plasmid recombination.

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          3731
COLE1     GTGAAACCATGAAAAATGGCAGCTTCAGTGGATTAAGTGGGGGTAATGTGGCCTGTACCC
PMB1      .C.....T.............G...G.......TT.AC...C........T...C...A
COLK      TGA...GATG......AAA...TG...TGA.G..T.CGCA..CA..ACCA..G.TATT.A
CLODF13   AGA..GT.GGT.....AA.GGCT.A.G.CATCCATTT.AC.TCA..ACATATGCTATG.A

COLE1     TCTGGTTGCATAGGTATTCATACGGTTAAAATTTATCAGGCGCGATCGC-G-CAGTTTTT
PMB1      ..........................................................G.........
COLK      CA...C..A....C...G.....................T..........G...-....
CLODF13   GT..CG........C..G...TA..A.........C.G......T.TC....GC.G....

COLE1     C-GGGTGGTTTGTTGCCATTTTTACCTGTCTGCTGCCGTGATCGCGCTGAACGCGTT-TT
PMB1      ..........................................................A.............
COLK      ..............................A..A.....................A....C
CLODF13   .C......G.......TTG.....T.CCGTA..C....GA.A...CGCC.GT..C..AC.

COLE1     AGCGGTGCGTACAATTAAGGGA--TTATGGTAAATCCACTTACTGTCTGCCCTCGTAGCC
PMB1      ....................................A.ACGTA...TAAT.TATCG.CAT
COLK      ..........................................--..................T..
CLODF13   G.....----..CGA.......TG...........ATCT.CGAGT.TCAGGTA.AA.AAA

             3969
COLE1     ATCGAGA
PMB1      ..GT.AC
COLK      .......
CLODF13   .G.TCCG
```

Figure 1.4 Sequence comparison of the cer site in ColE1, the ckr site in ColK, the crl site in CloDF13 and the analogous site in pMB1.

(·) represents homologous bases

(-) represents insertion of a gap

that the formation of multimers although not affecting the number of plasmid origins does reduce the number of independently segregating units in a cell. In a recombination proficient host the distribution of plasmid copy numbers among cells in a population about to divide will be asymmetrical with a "tail" of cells containing plasmids at a low copy number (Figure 1.3), from which plasmid free segregants will arise at a high frequency. Summers and Sherratt (1984), presented evidence in support of the origin counting hypothesis, showing that when the number of plasmid origins is increased per plasmid, a decrease in copy number and stability results. They also demonstrated a relationship between multimerization and instability for the unstable cloning vector, pACYC184.

Site-specific multimer resolution systems have been identified in a number of multicopy plasmids including ColE1 (Summers and Sherratt, 1984), ColK (Summers *et al*, 1985) and CloDF13 (Haakkart *et al*, 1984). Sherratt *et al* (1984) demonstrated that when a 282bp *Hpa*II-*Taq*I fragment of ColE1 is sub-cloned into pUC8, its stability is increased. This fragment contains the site, *cer* at which multimer resolution occurs. Sites analogous to *cer* are found in pMB1, ColK and CloDF13 (Figure 1.4). In pMB1 a region adjacent to the *Eco*RI restriction-modification genes has a 98% homology to *cer* over a 150bp core (3796-3946). There is a 90% homology between a 150bp region of *cer* and the *ckr* site in ColK (Summers *et al*, 1985). The *crl* site in CloDF13 exhibits 69% homology to the *cer* region. The similarities between these sites suggests that the mechanism of multimer resolution adopted by ColE1 may also be used by ColK, CloDF13 and pMB1. Site-specific recombination is used in two other systems to resolve plasmid multimers formed by homologous recombination i.e. the P1 *lox*P, *cre* system (Austin *et al*, 1981) and the FLP system of *S. cerevisiae* (Broach, 1982).

1.3 Recombination

Recombination involves the breaking and making of internucleotide bonds and can occur between separate DNA molecules (inter-chromsomal, inter-plasmidic) and sites in a single genetic determinant (intra-chromosomal, intra-plasmidic). When there is extensive homology between DNA sequences one gets DNA exchange cataylsed by the homologous recombination system, a key component of which is the recA gene product. Genetic exchange can occur in the absence of the recA gene product, albeit at a reduced frequency. There are two types of non-homologous events for which little sequence homology is required:

(i) Site-specific recombination - exchange at specific sites present in one or different DNA molecules.

(ii) Illegitimate recombination events - any event for which no alternative explanation can be found.

1.3.1 General recombination in relation to plasmid stability.

Recent work has demonstrated that plasmid multimerization leads to plasmid instability (Summers and Sherratt, 1984; Hakkaart et al, 1984; Austin et al, 1981; Jones, 1985). Under conditions where plasmidic homologous recombination is occurring, clones of cells will be generated, which possess high levels of multimers, a reduced number of segregating units and that will therefore have a high probability of generating plasmid free segregants (James et al, 1982; Summers and Sherratt, 1984).

E.coli has the capacity to promote general recombination between homologous DNA sequences. By the analysis of host mutations, Clark (1973, 1980) has defined the following pathways of homologous recombination:

(i) the RecA RecF pathway for plasmid recombination, which does not require functional recB, recC gene products (Laban and Cohen,

1981)

(ii) the RecA RecBC pathway for post-conjugational chromosomal recombination, while the RecA RecF pathway may play a minor role. Recent work by Amunsden et al (1986) has shown that exonuclease V previously thought to contain two polypeptides, the products of the recB and recC genes, also contains a polypeptide encoded by the recD gene. Mutants in the recB, recC genes can be indirectly suppressed by two mutations, either the sbcA mutation, which activates a third pathway the RecE pathway by derepressing synthesis of exonuclease VIII (Barbour, 1970) or the sbcB mutation, which activates the RecF pathway by inactivation of exonuclease I.

Many E.coli K-12 strains carry a prophage called Rac, which encodes the recE gene product, exonuclease VIII (Kaiser and Murray, 1979 and 1980). Hosts which are recBC sbcA e.g. JC 8679, have been found to be hyper-recombinogenic for plasmids. Analysis of plasmid recombination in these strains indicates that inter-plasmidic recombination in recBC sbcA strains is 2-5 times as frequent as in wild type E.coli strains (Laban and Cohen, 1981; James and Kolodner, 1983).

AB1157 derivative strains are extensively used in this work and are deleted for the Rac prophage. Consequently plasmid recombination cannot occur via the RecE pathway in these strains. The strain JC8679 (recBC sbcA) is an AB1157 derivative, which has acquired a copy of the Rac prophage in its construction, via mating from an Hfr strain carrying the sbcA23 mutation into a recBC AB1157 strain (Kaiser and Murray, 1980; Gillen et al, 1981).

Summers and Sherratt (1984) have looked at plasmid recombination of pACYC184 in a variety of E.coli strains. They found that pACYC184 was least stable and most multimeric in JC8679 (recBC sbcA), more stable in a rec^+ host and most stable in a recF host.

1.3.2 Site-specific recombination in general.

Site-specific recombination is a process requiring two defined sites and occurs independently of generalized recombination. The regions of homology required for site-specific recombination are usually much smaller than those required for general recombination. Site-specific recombinase genes are usually positioned adjacent to the site at which they act (Nash and Pollock, 1983) and promote reciprocal recombination between two limited DNA sequences. The recombination event does not involve the net synthesis or loss of any DNA and is a conservative process (Campbell, 1980).

The two sites which participate in the site-specific recombination events can be either identical, as is the case of the *res*, resolvase system of Tn3 (Grindley *et al*, 1982), or different as in the case of the lambda integration system (Mizuuchi *et al*, 1980). In the cases where the two sites are identical the recombination event will not change the sequence of the site, but in the case where the two sites are different, hybrid sites will be generated. Another feature of the sites is their asymmetry, which gives a directionality to the site-specific recombination reactions. If the two sites are in direct orientation to each other then the DNA between them is excised, whereas if the sites are in inverted orientation with respect to one another the DNA between them is inverted.

Site-specific recombination events are mediated either by a recombinase only, as is the case wih the *lox*P-*cre* system of P1 (Abremski *et al*, 1983) or with a recombinase and accessory factors, as seen for lambda integration, which requires *int* and IHF (Nash and Robertson, 1981). *In vitro* studies have shown that the requirements of site-specific recombination reactions, are simple, requiring a mono- or divalent cation and a buffer. There is no requirement for a high energy co-factor, but instead site-

specific recombinases behave a little like topoisomerases conserving the energy of the phosphodiester bond in a protein phosphate linkage and using that energy for the subsequent religation of the phosphodiester back bone. In all cases so far examined, the site-specific recombination reaction occurs in a stoicheiometric fashion, rather than catalytically. The systems in which this has been examined are the P1 loxP-cre system (Abremski and Hoess, 1984), the FLP system of the 2 micron plasmid of S. cerevisiae (Gronstajski and Sadowski, 1985), the lambda integration system (Nash and Robertson, 1981) and the Tn3 res resolvase system (Reed, 1981).

Three types of recombination events are promoted by site-specific recombination systems inter-molecular fusions, intra-molecular inversions and intra-molecular deletions (resolution). The choice of event is cruical to the biological function of each site-specific recombination system. A variety of biological function are carried out by site-specific recombination systems:

(1) integration and excision of bacteriophage from host genomes, e.g. lambda (Weisberg and Landy, 1983)

(2) resolution of cointegrate structures formed during transposition of e.g. Tn3 and gamma-delta (Arthur and Sherratt, 1979)

(3) changing the host range of bacteriophages e.g. Mu gin system (Van de Putte et al, 1980)

(4) switching of the flagellar antigen of Salmonella typhimurium by the hin system (Iino and Kutsukake, 1980)

(5) control of type I fimbriation in E.coli (Diderichsen,1980; Klemm, 1986)

(6) maintenance of high copy number plasmids in E.coli (Summers and Sherratt, 1984) and in S.cerevisiae (Broach, 1982; Broach et al, 1982)

(7) diversity of antibodies (Bernard et al, 1978) and of T-cell receptors of the immune system (Clark et al, 1984)

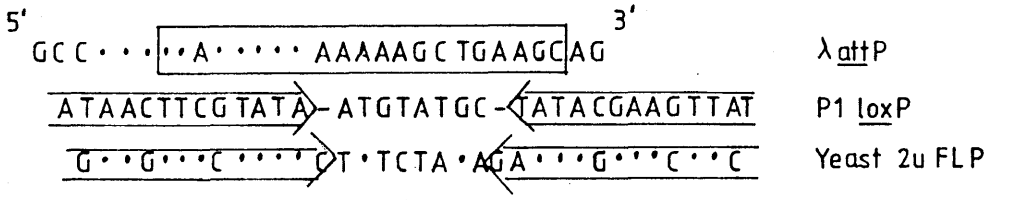


Figure 1.5 Sequence comparison of the 13bp inverted repeats of loxP site with the lambda attP site and the yeast 2 micron FLP site.

▭ represents the region where crossover occurs

⟹ represents inverted repeats

(-) represents gaps

(·) represents homologous bases

(Hoess and Abremski, 1984)

These examples demonstrate the wide variety of biological functions, which site-specific recombination systems carry out.

1.4 Complexity of site-specific recombination systems.

Different site-specific recombination systems vary enormously in their complexity of requirements. This variety in complexity of requirments is illustrated using the following examples.

1.4.1 P1 loxP-cre system.

This system is one of the least complex site-specific recombination systems. P1 in the prophage state behaves as an autonomously replicating plasmid and is maintained at one to two copies per cell (Ikeda and Tomizawa, 1968). Less than one in 10^4 E.coli cells containing P1 produces a cured cell (Rosner, 1972). The efficient maintenance of P1 is due to a site-specific recombination system encoded by P1, which converts any dimers formed to monomers and so ensures effective partition. The requirements of this system are a P1 recombinase encoded by the cre gene and a site on P1 called loxP (Austin et al, 1981). Nuclease protection studies have revealed that Cre is in contact only with loxP and that loxP contains two protein-binding domains, located within 34bp (Hoess and Abremski, 1984). The directionality of this system is dictated by the asymmetry in the core region of the loxP site. The loxP-cre results are in stark contrast to other systems such as lambda and Tn3. In these systems the recombinase has multiple binding sites, some of which are distant to the crossover site. Although the P1 system is simplistic in comparison to other systems the site shares some homology to att and the FLP site in the yeast 2 micron plasmid (Figure 1.5). The 13bp inverted repeat sequence of loxP (Hoess and Abremski, 1984) is homologous to the lambda att site (Landy

and Ross, 1977) and to the yeast 2 m FLP site (Broach et al., 1982). This homologous 13bp is part of the sequence of the core of the core of att recognized by int. The sites and recombinases of these systems are unlikely to be interchangeable but are probably related in an evolutionary manner.

1.4.2 Lambda integration system.

The requirements of the integration system for lambda are more extensive than those required by P1. This is a reciprocal recombination event between specific sites on the phage and in the host chromosome, attP(240bp) and attB(25bp) (Nash, 1981). Each att site has a core of 15bp, flanked by two arms. It is within the core region that exchange of DNA strands occurs. When integration of lambda occurs two hybrid sites are formed, attR and attL, excision occurs by a reciprocal recombination event, which requires the product of the xis gene (Nash, 1975).
Lambda integration requires two proteins phage encoded Int, which is a polypeptide of 40kd and a host encoded protein IHF, which consists of two polypeptides α and B. The α polypeptide is 11kd and coded by the himA gene, whereas the B polypeptide is 9.5kd and is coded for by the himD gene (Kikuchi et al, 1985; Flamm and Weisberg, 1985; Miller and Nash, 1981). Efficient integration requires Int and IHF, however Int alone is capable of promoting integration of lambda at a very reduced frequency (Lange-Gustafsson and Nash, 1984), indicating that IHF acts as an acessory protein, enhancing the activity of Int. Nuclease protection studies have revealed that IHF binds to three distinct sites in attP and that these sites are interspersed with four Int binding sites (Craig and Nash, 1984). The role of IHF in this system is unclear, perhaps it functions to facilitate the formation of higher order structures by protein-DNA interaction or by producing bends in the DNA (Wu and Crothers, 1984).

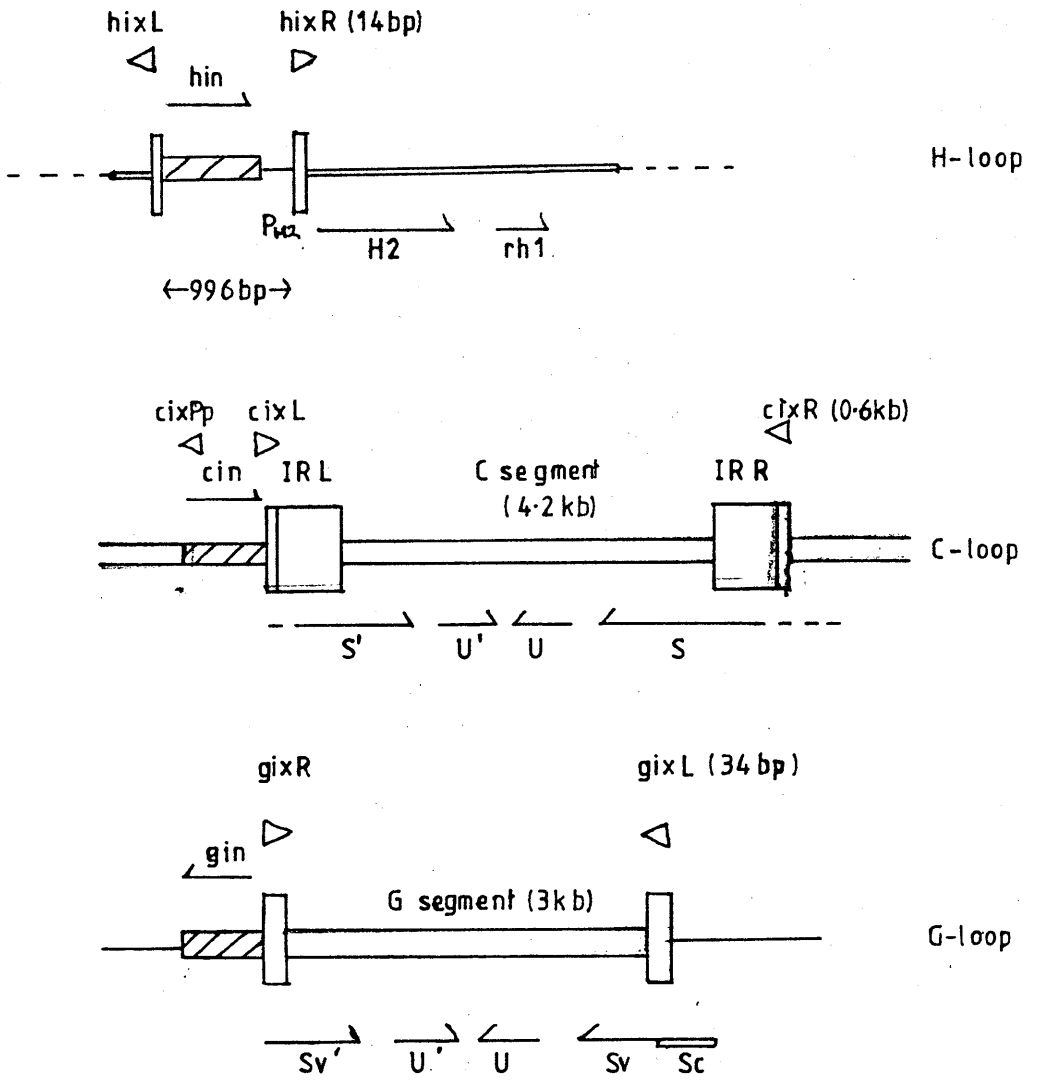


Figure 1.6 Comparison of the Salmonella H-loop, P1 C-loop and the Mu G-loop inversion systems.

The H-loop system requires host factors - Factor II and HU and utilizes a 60bp recombinational enhancer sequence in the hin gene. The G-loop system requires a host factor called FIS and utilizes a recombinational enhancer sis (Hiestand-Nauer and Iida, 1983).

These site-specific inversion systems serve to allow alternative expression of two sets of genes.

1.4.3 Inversion Systems.

Inversion systems are utilized by phage Mu to change its host range (Simon *et al*, 1980), by phage P1 and P7 to alter their host range (Chow and Bukhari, 1976; Craig, 1985) and by *Salmonella typhimurium* to alter its flagellar antigens (Iino and Kutsukake, 1980). These site-specific inversions are used as a biological switch.

The H-loop system of *Salmonella typhimurium* requires a recombinase, which is encoded by the *hin* gene and two host factors, called FactorII and HU (Johnson and Simon, 1985; Johnson *et al*, 1986). The requirement for host factors was discovered when an *in vitro* system was set up. This inversion system acts to connect or disconnect the flagellin promoter for phase two flagellin and for a repressor rh1 of phase one flagellin (Figure 1.6) (Zieg *et al*, 1977). This system also utilizes a recombinational enhancer sequence of 60bp, which is within the *hin* coding sequence. The presence of the enhancer sequence increases recombination twenty fold. What does the enhancer sequence do? There are many possibilities including that interaction of Hin at the enhancer sequence may produce a conformational change, which could increase the activity of Hin by increasing the acessibilty of Hin to the substrate or that the enhancer site acts as a wrapping site for the formation of a complex between Hin, enhancer and the recombination sites (Johnson and Simon, 1985). These enhancer sequences are similar to eukaryote transcriptional enhancers (Serfling *et al*, 1985) in that they act in *cis* to enhance recombination at a distance and in an orientation independent fashion. The related system in the bacteriophage Mu also involves a host factor, FIS (factor for inversion stimulation) and a recombination enhancer called *sis*, which increases recombination fifteen fold (Kahmann *et al*, 1985). The requirement for host factors in the Mu *gin* system (Kahmann *et*

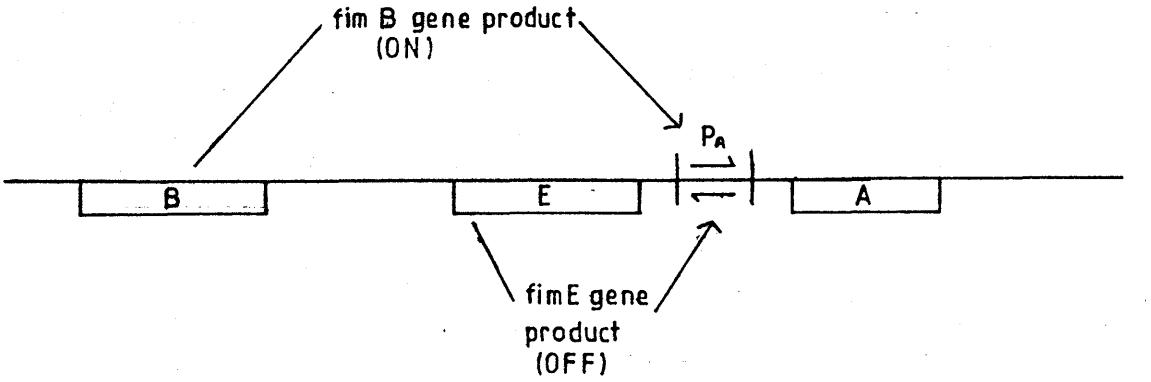


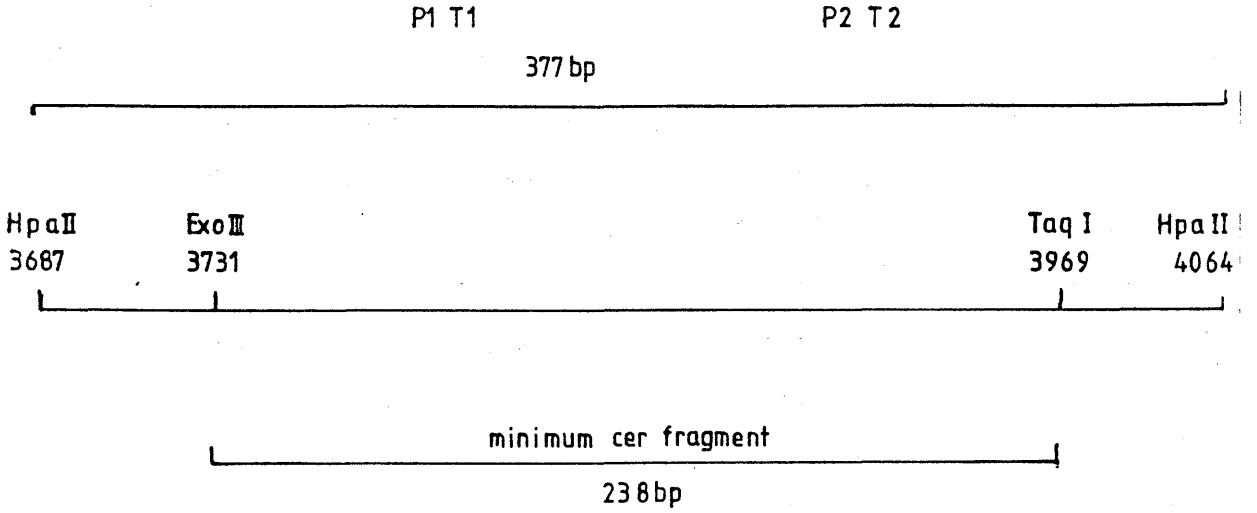
Figure 1.7 Organization of the fim genes.

The fimB gene product controls the switching of the 300bp region into the on position with the promotor pointing into fimA, whereas the fimE gene product is responsible for switching the 300bp region into the off position with the promoter pointing away from fimA.

al, 1985) and in the Salmonella hin system (Johnson and Simon, 1985) were discovered when setting up in vitro recombination systems. When the recombinases for these systems were purified away from host components the recombination frequency was found to decrease, but when the host component was added back the recombination frequency was enhanced. The gin and hin systems are very similar to the P1 cin system, the recombinases exhibiting a 60-70% amino acid homology (Johnson et al, 1986), hence there is a strong possibility that a host factor will also be required for the cin system.

1.4.4 Type I fimbriation.

A site-specific inversion system controls type I fimbriation in E.coli. Cells are either completely fimbriated or bald. This is due to a periodic inversion of a 300bp DNA sequence, which contains the promoter for the fimbria subunit gene, fimA. The inversion is controlled by the gene products of two genes fimB and fimE, located upstream of fimA (Figure 1.7). The fimB gene product controls the switching of the 300bp region into the on position, with the promoter pointing into fimA, whereas fimE is responsible for switching the 300bp region into the off position, with the promoter pointing away from fimA (Klemm, 1986). The fim genes map to 98mins on the E.coli K-12 genetic map. It has been suggested that the flu gene, mapping to 43mins (Diderichsen, 1980) encodes the recombinase and that the fim B and fimE gene products control the direction of the process. The control of type I fimbriation is closely linked to the control of expression of the fimB and fimE genes, exactly how is as yet unknown.



	Mutant + Mutant	WT + Mutant
731 to 3969	+	+
735 to 3969	+/−	+
746 to 3969	−	+
790 to 3969	−	−

Figure 1.8 The organization of the cer fragment: defining the minimal functional cer site using ExoIII deletions.
These deleted cer fragments were analysed for cer-specific recombination by cloning them into a pseudo-cer dimer with an identical copy or a wild type cer fragment (280bp).

1.4.5 The *cer* system of ColE1.

What are the requirements of the ColE1 *cer* site-specific recombination systems in relation to the other systems discussed? This system acts to resolve multimers to monomers and hence to increase the stability of ColE1, by maximising the number of independently segregating units. Similar resolution systems also exsist in other high copy number plasmids, such as ColK, CloDF13 and pMB1. The site *cer* at which resolution occurs is contained in a 282bp *Hpa*II-*Taq*I fragment. Sites that are homologous to *cer* have been identified in pMB1, ColK and CloDF13. These sites are respectively 98%, 90% and 69% homologous to a 150bp core region of *cer*, which extends from 3796 to 3946bp (Summers and Sherratt, 1985). The divergence of sequence homology reflects the structural and functional relationships of these plasmids.

(A)The *cer* site.

The original fragment containing the *cer* site was a 377bp *Hpa*II fragment running from 3687 to 4064bp (Summers and Sherratt, 1984). The functional *cer* fragment was redefined (Sherratt *et al*, 1984) as a 282bp *Hpa*II-*Taq*I fragment (3687-3969bp). David Summers has further redefined the functional *cer* site using *Exo*III deletions of the *Hpa*II-*Taq*I fragment. These deleted *cer* fragments were analysed for *cer* specific recombination by cloning them into a pseudo-*cer* test plasmid with an identical *cer* or a wild type *Hpa*II-*Taq*I *cer* fragment (Figure 1.8). Using *Exo*III deletions the *cer* site has been minimized to a 238bp fragment, which in a pseudo-*cer* dimer construct will recombine with another copy of itself at normal rates. If a further 4bp are deleted from co-ordinate 3731 to 3735bp then this fragment will recombine with a copy of itself at reduced rates but will recombine with a wild type copy at normal rates. If a further 11bp are deleted

then the mutant cer will no longer recombine with itself but will recombine with a wild type copy. When an additional 44bp is deleted to co-ordinate 3790 then the mutant cer will no longer recombine with a copy of itself or a wild type copy.

The reasons for this gradual loss of activity are as yet unknown, but could be due to a requirement for an enhancer sequence, or requirements for binding sites for accessory host factors in cer. The deleted cer running from 3746 to 3969bp can recombine with a wild type cer but not with a copy of itself. This could be explained by suggesting that the wild type cer site provides in trans a factor absent in the deleted cer.

Computer analysis of the cer fragment reveals two promoters P1 and P2 and two terminators T1 and T2 (Figure 1.8). The two promoters are highly conserved in ColK and pMB1 but less so in CloDF13. In the CloDF13 crl region there is some divergence at P2. The -10 region of P1 is highly conserved in ColK, pMB1 and CloDF13, although the -35 region is not as well conserved. T1 and T2 are conserved in ColE1 , ColK and pMB1 but not as well in CloDF13. Some of the transcription control signals are conserved in ColE1, ColK, pMB1 and CloDF13, which may imply that the region of the site is highly conserved, which would in turn conserve the transcription control signals. Transcription in cer is being investigated by David Summers.

The crossover region was identified to a 35bp region in cer from 3906 to 3942bp, by sequencing of cer/ckr hybrid sites (Summers et al, 1985). The crossover region is only 35bp in length and yet the minimum cer fragment is 240bp in length, implying that sequences in addition to the crossover site are required. Perhaps the cer fragment encodes an RNA or polypeptide species, that is involved in cer-specific recombination. One ORF is conserved in cer and ckr, which could encode for a polypeptide of 53 amino acids and is not conserved in CloDF13. Is this ORF conserved in ColE1 and ColK or is it the region of the site which

is conserved? The functional cer fragment may be larger than the crossover region as it contains binding sites for accessory factors as seen in the lambda integration system (Craig and Nash, 1984).

(B) Origin of the cer-specific recombinase.

The recombinase, which acts at cer was initially thought to be encoded by ColE1. Site-specific recombinases are usually positioned adjacent to the site at which they act hence the most likely ORFs are the cer adjacent ones, however preliminary experiments suggest that this is not the case. Host mutants, which affect cer-specific recombination were constructed (Chapter 4), allowing us to define at least two host complementation groups required for cer-specific recombination. The genes, which code for these host factors are called xer genes. These host functions also appear to be necessary for site-specific recombination at the ckr site of ColK and the crl site of CloDF13 (Chapter 4). Implying that similar site-specific recombination systems may be used by other high copy number plasmids and that these systems may rely on xer gene products.

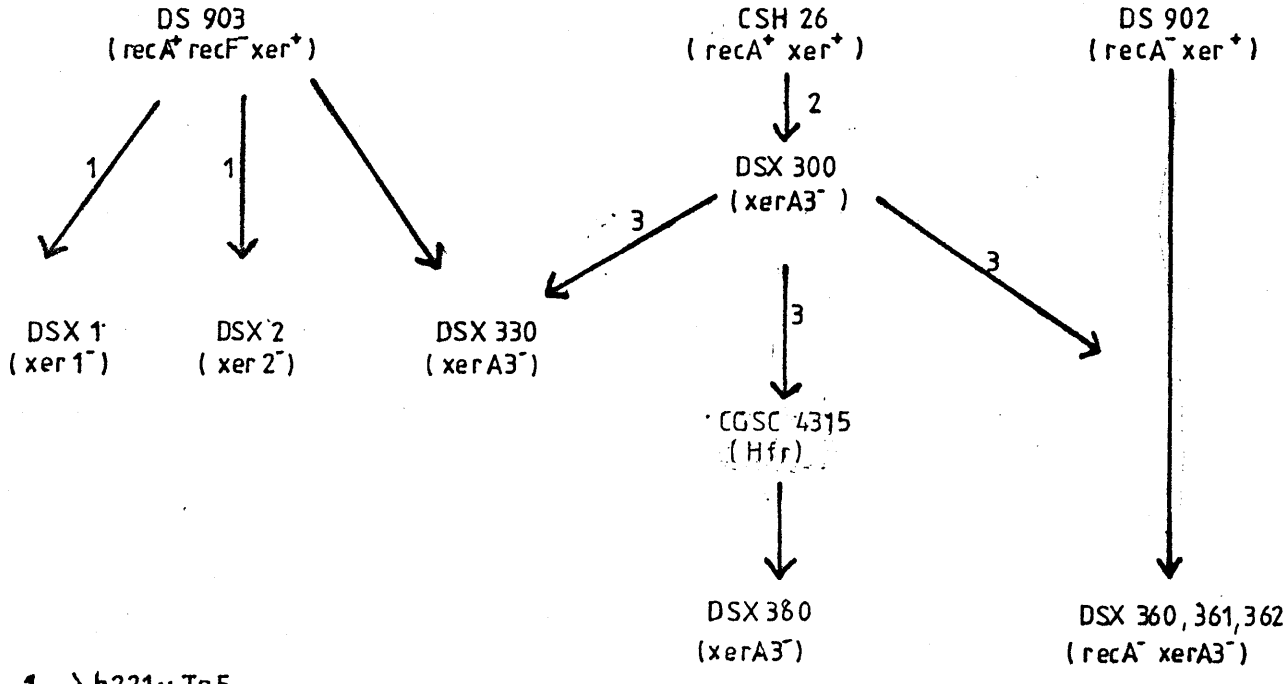
The requirement for more than one host factor has been observed in other site-specific recombination systems, such as the lambda integration system, the type I fimbriation system and some of the inversion systems. The role of these host factors in cer-specific recombination are as yet unknown but one could postulate that one may function as the recombinase and that the other may be an accessory factor, perhaps involved in bending of DNA (Wu and Crothers, 1984) or in assisting in the formation of higher order protein-DNA structures.

CHAPTER 2

MATERIALS AND METHODS

Table 2.1A BACTERIAL STRAINS

Strain	Characteristics	Reference/Source
DS.903	*recF*143 *thi* *leuB*6 *his*4 *thr*1 *proA*2 *argE* *galK*2 *supE*44 *tsx*33 *rpsL*31 *ara*14	Horii & Clark (1973) (AB1157)
DS.902	As DS.903 but *recA*13 *argE*$^+$ *recF*$^+$	Howard-Flanders (1966) (AB2463)
JC.8679	As DS.903 but *recBC* *sbcA* *recF*$^+$	Clark
DS.801	As DS.903 but *recA*$^+$ *recF*$^+$	D. Sherratt
DS.882	WB.882 *E. coli* C *trp*	D. Sherratt
DS.807	CR63/1	D. Sherratt
DS.803	*E. coli* B *rpsL*31	D. Sherratt
CSH.26	*thi* *ara* *rpsL* Δ(*pro-lac*)	Miller (1972)
CB.50	*rpsL* *ara* *thi* Δ(*pro-lac*)	C. Boyd
CB.51	As CB.50 but *dam*	C. Boyd
W5445	*pro* *leu* *thr* *thi* *supE* *tonA* r$^-$ m$^-$	S. Primrose
W5445/A	As W5445 but *recA*13	Chapter 4
KL-16	*srl*::Tn10 *recA*13	Kleckner (1977)
CB.1	*galK*	M^{c}Kenny
DS.941	As DS.903 but *lacZ* ΔM15	D. Sherratt
CGSC.4315	Hfr PO18 of Hfr 14 *thi*1 *malB*16	Bachman (KL209)
CGSC.4241	Hfr PO48 *malB*28 *sfa*4	Bachman (Ra-2)
CGSC.4311	Hfr PO2A of Cavali Hfr *relA* *tonA*22	Bachman (KL226)
CGSC.5051	Hfr PO120 *lacY* *xyl*7 *ara*41 *mtl*2	Bachman (Hfr-P801)
DSX.380	As CGSC.4315 but Kan^r *xerA*	Chapter 6
F'4258	PO18 of Hfr J4 (Chapter 6) (host strain JC1553 *recA* *metB*1)	Bachman
F'4260	PO48 of Ra-2 (Chapter 6) (host strain JC1553 *recA* *metB*1)	Bachman
F'4315	F' derived from cross CGSC.4315 x DS.902	Chapter 6
F'4241	F' derived from cross CGSC.4241 x DS.902	Chapter 6
F'4315.X	F' derived from cross DSX.380 x DS.902	Chapter 6
DSX.1	As DS.903 but *xer*1	Chapter 4
DSX.2	As DS.903 but *xer*2	Chapter 4
DSX.330	As DS.903 but *xerA*3	Chapter 4
DSX.300	As CSH.26 but *xerA*3	Chapter 4
DSX.360	As DS.902 but *xerA*3	Chapter 5



1 λb221:: Tn5

2 λ467 :: Tn5

3 P1 transduction

Table 2.1B Derivation of xer Mutants

Table 2.2 PLASMIDS

Plasmid	Description	Phenotype	Size(kb)	Reference/Source
ColE1	naturally occurring	$Ce1^+$ $Ie1^+$	6.6	D. Sherratt
pBR322	derived from pMB1	Ap^r Tc^r	4.316	Sutcliffe (1978)
pACYC184	derived from p15A	Cm^r Tc^r	4.0	D. Sherratt
pAT223	derived from pKK223	Ap^r	3.88	C. Boyd (Chapter5)
pUC8	derived from pBR322	Ap^r	2.67	Vieira & Messing (1982)
pUC19	derived from pBR322	Ap^r	2.67	Yanish-Perron (1985)
pKS450	pUC9 + HpaII cer fragment	Ap^r	3.07	D. Summers (Chapt.3)
pKS451	pUC9 + HpaII cer fragment	Ap^r	3.07	D. Summers (Chapt.3)
pKS440	derived from pKS450 contains 2 cer sites in direct repeat	Ap^r	3.36	D. Summers (Chapt.3)
pKS441	derived from pKS440 Cm^r gene between cer sites	Ap^r Cm^r	4.66	D. Summers (Chapt.3)
pGS500	derived from pACYC184 contains 2 cer sites in direct repeat	Cm^r Tc^r	5.48	(Chapter 5)
pCS202	derived from λDV plasmid contains 2 cer sites in direct repeat	Cm^r Tc^r	7.7	(Chapter 3)
pRY10	derived from pUC8 contains ckr site from ColK	Ap^r	3.08	S. Yaish (1985)
pEV87	contains crl site from CloDF13	Ap^r	3.1	Hakkaart (1984)
ppE14	derived from pACYC184 contains recA gene	Cm^r	7.5	Emmerson
pKS200	ColE1 + Ap^r gene from Tn3	Ap^r	8.2	D. Summers
pKS220	ColE1 - HaeIIB,D fragments + Ap^r gene from Tn3	Ap^r	6.2	D. Summers
pCS300	mutant xerA gene in pBR322 at EcoRI site	Ap^r	19.5	C. Stirling (Chapter 5)
pGS30	wild type xerA gene in pAT223 on HindIII EcoRI fragment	Ap^r	12.3	Chapter 5
pGS31	wild type xerA gene in pAT223 on EcoRI fragment	Ap^r	13.1	Chapter 5
pGS35	pAT223 + flanking chromosomal DNA	Ap^r	6.2	Chapter 5
pGS36	pBR322 + 3.1kb SphI fragment	Ap^r	7.4	Chapter 5
pGS37	pBR322 + 3.0kb SphI fragment	Ap^r	7.3	Chapter 5
pGS38	pUC19 + 0.95kb AccI-SphI fragment	Ap^r	3.65	Chapter 5

Table 2.3 BACTERIOPHAGES

Phage	Comments	Source/Reference
P1	Used in P1 transductions	A. Jessop
λ::Tn5	*rex*::Tn5 b221	D. Summers
λ::467	*rex*::Tn5 b221 *c*I857 *O*am29 *P*am80	N. Kleckner
λvir	mutant in O_L & O_R, grows on lambda lysogens	D. Sherratt
λcI	produces no *c*I repressor and can not grow on a lambda lysogen	D. Sherratt

Table 2.4

Chemical	Source
General chemicals and organic chemicals	B.D.H., Koch-light Laboratories, Hopkins and Williams, B.C.L., May and Baker
Antibiotics	Sigma
Biochemicals	Sigma
Media	Oxoid, Difco
Agar	Oxoid, Difco
Agarose and LMP agarose	B.R.L.
SDS	Serva
Radiochemicals	Amersham International
Transfer membranes	Pall (Biodyne A)
Sephadex G-50	Pharmacia Fine Chemicals
Benzyl penicillin	Glaxo
"Replicote"	Hopkins and Williams
X-gal	B.R.L.

Enzymes

All the enzymes used were obtained from B.R.L., with the exception of the following:-

Calf-Intestinal Alkaline Phosphatase - B.C.L.

Lysozyme - Sigma

Ribonuclease A - Sigma

Table 2.5

Antibiotics

Antibiotic	Selective concentration	Stock Solution
Ampicillin	50ug/ml	5mg/ml in H_2O
Chloramphenicol	50ug/ml	5mg/ml in EtOH
Streptomycin	50ug/ml	10mg/ml in H_2O
Naladixic acid	20ug/ml	2mg/ml in 1M NaOH
Rifampicin	50ug/ml	5mg/ml in Methanol
Tetracycline	10ug/ml	1mg/ml in H_2O
Kanamycin	25ug/ml	5mg/ml in H_2O

2.1 Bacterial Strains

The bacterial strains used were nearly all *Escherichia coli* K-12 derivatives, with the exception of an *E. coli* B and an *E. coli* C strain. These strains are listed in Table 2.1A
Genotype and phenotype symbols are those recommended by Bachmann *et al* (1976) and Novick *et al* (1976).
The derivation of the *xer*$^-$ strains is summarized in Table 2.1B.

2.2 Plasmids

The plasmids used in this study are listed in Table 2.2.

2.3 Bacteriophage

The bacteriophage used are listed in Table 2.3. Symbols for genotype and phenotype are those recomended by Bachmann *et al*, (1976).

2.4 Chemicals

The chemicals used and their sources are listed in Table 2.4.

2.5 Culture Media

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

L-agar: as above L-broth containing 1.5% agar

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

Minimal Agar: as above Minimal broth containing 17.5g/l of agar. When required supplements were added to minimal media at the following concentrations:-

Arginine, Histidine, Methionine, Threonine: 100ug/ml

Proline, leucine: 40ug/ml

Thiamine (B1): 20ug/ml

Glucose: 120ug/ml

Xylose: 2,500ug/ml

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

Soft Agar: 6g of agar in 1l of distilled water.

Davis-Mingioli Salts (x4): 28g K_2HPO_4, 8g KH_2PO_4, 4g $(NH_4)_2$, 1g sodium citrate, 0.4g $MgSO_4.7H_2O$, made up to 1 litre with distilled water.

D/M Minimal Media: 25ml D/M salts, 5ml 20% cas-amino acids, 1ml 20% glucose, 0.5ml vitamin B1 (1mg/ml), made up to 100ml with water.

Phage Buffer: 7g Na_2HPO_4, 3g KH_2PO_4, 5gNaCl, 0.25g $MgSO_4$, 15mg $CaCl_2.2H_2O$, 1ml 1% gelatin made up to 100ml with distilled water.

2.6 Sterilization

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

2.7 Buffers and other solutions

(i) General buffers

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

MC buffer: 0.1M $MgSO_4$, 0.005M $CaCl_2$

Calcium chloride solution: 50mM $CaCl_2.2H_2O$.

Magnesium sulphate solution: 100mM $MgSO_4.7H_2O$.

(ii) Electrophoresis

10 x E Buffer: 48.4g Tris, 16.4g NaAc, 3.6g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH adjusted to 8.2 with acetic acid.
10 x TBE Buffer: 109g Tris, 55g Boric acid, 9.3g $Na_2EDTA.2H_2O$, made up to 1 litre in distilled water, pH is 8.3.

Tris/glycine Running Buffer (protein gels): 14.41g glycine, 3.03g Tris HCL, 1g SDS, made up to 1 litre with distilled water.

DNA Final Sample Buffer (FSB): 10% Ficoll, 0.5% SDS, 0.05% Bromophenol blue, 0.06% Orange G, in buffer E.

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

Protein Final Sample Buffer: 50% glycerol, 5% SDS, 5% B-Mercaptoethanol (added fresh before using), 0.01% Bromophenol

blue, in 50mM Tris HCL pH 6.8.

Upper Buffer for SDS-PAGE gel (4x): 0.5M Tris HCL, pH 6.8, 0.4% SDS.

Lower Buffer for SDS-PAGE gel (4x): 1.5mM Tris HCL, pH8.8, 0.4% SDS.

(iii) DNA Manipulation

Restriction Buffers:

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

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

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

10x Core Buffer: supplied by B.R.L., stored at -20°C.

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

ATP For Ligations (4mM): 4mM ATP in 4mM Tris-HCL pH 7.5; stored separately from ligation buffer at -20°C.

1x TE Buffer: 10mM Tris-HCL, 1mM $Na_2EDTA.2H_2O$, pH 7.6.

(iv) DNA Hybridization

20 x SSC: 3M NaCl, 0.3M Na Citrate, pH 7.0.

20 x SSPE: 3.6M NaCl, 0.2M NaH_2PO_4/Na_2HPO_4, 20mM Na_2EDTA, pH 7.0.

50 x Denhardts solution: 1% Ficoll, 1% Polyvinylpyrolidane, 1% Bovine serum albumin.

Pall Denaturing Buffer: 2.5M NaCl, 0,5M NaOH.

Pall Neutralizing Buffer: 3M NaAc pH 5.5.

Pall Wash Buffer: 5mM Na phosphate, pH 7.0, 1mM EDTA, 0.2% SDS.

Pall High Stringency Wash Buffer: 5mM Na phosphate pH 7.0, 1mM EDTA, 0.1% SDS, 0.1x SSC.

Pall Low Stringency Wash Buffer:5mM Na phosphate pH 7.0, 1mM EDTA, 0.1% SDS, 2x SSC.

(v) DNA Preparation Solutions

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

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

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

Sucrose solution: 25% sucrose, 50mM Tris-HCL pH 8.0.

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

STET Buffer: 50mM Tris-HCL pH 8.0, 50mM Na_2EDTA pH 8.0, 5% Triton X-100, 8% sucrose.

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

Crystal violet: this indicator was used to screen for the presence or absence of Cm resistance (Proctor and Rownd, 1982). After incubation at 37°C, Cm^r colonies were purple and Cm^s colonies were white. Crystal violet was made up at 4mg/ml in distilled water and used at 4ug/ml in L-agar, and 1.8ug/ml in minimal agar (Chapter 4).

2.9 Growth Conditions

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

Growth on plates was on L-agar, isosenstest or minimal media plus supplements. Antibiotics were added as required. Plates

contained 25ml of L-agar solution and were incubated for approximately 15 hours at 37°C. All dilutions for plating out cells were carried out in phage buffer.
Bacterial strains were stored in 50% L broth, 40% glycerol at -20°C. Inocula from these were applied to selective plates and after growth at 37°C single colonies were selected for use.

2.10 *In Vivo* Techniques

Transformation of *E.coli*: Plasmids were introduced into different host strains by genetic transformation. *E.coli* is not naturally competent to take up DNA, but can be made to do so by treatment with $CaCl_2$. This makes a proportion of the cell population competent. An overnight culture of the recipient strain was diluted 1 in 100 into 20ml of L broth and was grown to mid-log phase 1 x 10^8cells/ml (90-120mins). The cells were harvested by centrifugation at 10K (12000g) for 5mins at 4°C and then washed by re-suspending in 10ml of cold (4°C) 50mM $CaCl_2$. The cells were pelleted again, re-suspended in 0.6mls of cold 50mM $CaCl_2$ and kept on ice for at least 15mins before being used. DNA in TE buffer was then added to 200ul aliquots of $CaCl_2$ treated cells and the mixture was kept on ice for 15mins. The cells were then heat shocked for 5mins at 37°C and returned to ice for a further 15mins. An equal volume of L broth was added to the cells prior to incubation at 37°C, in order to allow expression of plasmid genes. The standard expression time was 90mins, although Tc^r plasmids were given 120mins. Aliquots of the transformation mixture were then spread onto selective plates.

Fmatings were always carried out on the surface of an L-agar plate. Unless otherwise stated the inocula of recipient and donor were taken from a single colony. The mating was set up by mixing 5ml of stationary phase recipient and 5ml of exponential phase donor. This mixture was concentrated ten fold by centrifugation and allowed to absorb onto the surface of a dry L-agar plate. Mating was allowed to proceed for 3hours at 37°C to ensure transfer had occurred. The cells were then washed off the plates with 1ml of phage buffer and dilutions were spread onto selective plates.

Interrupted Liquid Matings: These matings are carried out to determine the time of entry of particular markers on the E.coli K-12 chromosome, as Hfr strains transfer markers in a linear fashion from a fixed point of origin. The donor and recipient stains were grown until they reach the exponential phase of growth (2×10^8cells/ml) and then mixed in the ratio of 1Hfr: $10F^-$. This mating mixture is incubated in a 37°C water bath. Samples are removed at different time points and the mating pairs are disrupted by vortexing, diluting and then plating out on selective media. Naladixic acid was routinely used as the male contra selection and to inhibit conjugal transfer after plating (Bouck and Adelberg, 1972).

Preparation of P1 lysates: The donor strain was grown to mid-log phase in L broth, the bacteria were then pelleted at 10K(12000g) and resuspended in a small volume of R broth plus $CaCl_2$ and 100mM $MgSO_4$. Sufficient phage to produce an m.o.i. of 0.001 were added to the bacteria and allowed to absorb for 20mins at 37°C. 500ul of infected cells were added to 3mls of top agar and this was poured over a thin wet L-agar plate. These were then incubated in a sandwich box containing a small beaker of water for 6-8hours only. At this time substantial lysis of the bacteria had

occurred. The lysate was scraped off the plate with a glass spreader and placed in a centrifuge tube. A few drops of chloroform was added and the lysate was vortexed vigorously for 30sec. This was allowed to stand for 30mins at room temperature and then the debris was pelleted by spinning at 10K(12000g) for 10mins. The resulting supernatant contains the P1 phage particles and was subsequently titred to determine the number of phage.

P1 transduction: The recipient strain was grown to mid-log phase (2×10^8cells/ml) in L broth plus 10mM $CaCl_2$ and the cells pelleted at 10K(12000g) for 10mins. Sufficient phage to give an m.o.i. of 0.01-0.1 were added to 200ul aliquots of bacteria in MC buffer. This mixture was incubated at 37°C for 20mins, then the cells were pelleted and washed twice in 1M Na citrate. These cells were then plated in 3mls of soft agar on selective plates and incubated at 37°C for 48hours.

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

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

Initially all colonies are dark-yellowish brown, however, p^+ colonies soon begin to turn white and the stained agar surrounding the colony begins to clear, until a "halo" around the

colony is formed. This halo increases in size with time. In contrast, plasmid free colonies remain brown in colour and no localized clearing occurs. After 15mins, there is a general clearing of the media.

To obtain optimum results with this technique, it is advisable to have less than 300 colonies per plate, otherwise clearing around one colony may also affect adjacent colonies. Finally isosenstest agar is used in preference to L-agar because it contains soluble starch which is required for the blue-black coloration.

Stability Testing: A single colony was used to innoculate 2.5ml Of L broth which contained appropriate antibiotics to select against plasmid-free cells. After overnight incubation, the culture was diluted 10^{-6} fold into D/M minimal medium and grown into stationary phase. The cycle of dilution and growth was performed 5 times and each cycle represented 20 generations ($2^{20}=1x10^{6}$). From each stationary phase culture, samples were diluted and plated out onto isosenstest agar and the resulting colonies were tested for plasmid content via the B-Lactamase plate assay (see above). Around 1000 colonies were tested every 20 generations and after 100 generations the limit of detection should be 10^{-5} per cell per generation.

Testing recA phenotype by U.V. sesitivity: The recA phenotype of strains was determined by testing the sensitivity of that strain to ultra-violet light. Mid-log phase cultures of the strains to be tested were diluted from 10^{0} to 10^{-6} (recA$^{+/-}$ strains were always included as controls). Then 10ul spots of each dilution were placed on three replicate L-agar plates and allowed to dry. The control plate was not exposed to U.V. light, whereas the other two were exposed to the U.V. light (254nm,42cm from the source: 120uWcm^{-2}) for 30sec and 60sec. These plates are then incubated overnight at 37^{o}C in the dark. The recA^{-} strains grow

on the control plate but not on the 30 and 60sec exposure plates, whereas the *recA*$^+$ strains grow on the control plates and on the plates exposed to U.V. light.

λ::Tn5 mutagenesis: An overnight culture of the strain to be mutagenized was grown up overnight in L broth with added $MgSO_4$(10mM). This culture was diluted 1:100 in the same broth and grown to mid-log phase with shaking. Then 0.5ml-1ml aliquots of the culture were incubated with λ::Tn5 at a m.o.i. of between 1 and 10 for 15mins. The cells were then harvested by centrifugation at 10K (12000g), 4°C, washed twice in L broth and resuspended in L broth. 100ul aliquots were then spread on appropriate selective plates.

Testing for colicin production: The strain to be tested was stabbed into a nutrient plate and a control plate. These plates were then incubated overnight at 37°C. The cells on the test plate were killed by exposure to chloroform vapour for 10mins. The plates were overlaid with 2.5ml of soft agar at 46°C, containing 0.2ml of stationary phase colicin sensitive bacteria and reincubated overnight at 37°C. Colicin production was detected as a zone of killing in the indicator lawn around the stab.

2.11 *In Vitro* techniques.

Cleared lysate DNA preparation: 200ml cultures of selectively grown plasmid containing cells were pelleted at 10K (12000g) at 4°C for 5mins. The pellet was resuspended in 3.3ml of cold 25% sucrose 50mM Tris pH 8.0 and incubated on ice for 10mins. Then 1.3ml of 250mM Na_2EDTA pH 8.0 was gently mixed in and incubated on ice for a further 10mins. The protoplasts were lysed by the addition of 5.3ml of lytic mix solution mixed by inversion and

incubated on ice for 30mins. The crude lysate was cleared by centrifugation at 18K (39200g) at 4°C for 30mins. This separated the cell debris as a spongy pellet form the supernatant, which contained the plasmid DNA. The plasmid DNA was further purified by CsCl/EtBr gradient centrifugation: 5g of CsCl was dissolved in 4.83ml of cleared lysate and 0.33ml EtBr(3mg/ml), producing a density of 1.58g/ml. The mixture was poured into a 10ml polypropolene ultra-centrifugation tube, topped up with paraffin oil and centrifuged at 49K (200000g) at 15°C for 16 hours. Two bands were visible, a lower supercoiled plasmid band and an upper chromosomal band. The plasmid specific band was removed with a hypodermic syringe, by puncturing the tube at the appropriate point. The EtBr was removed by repeated extraction with butanol and the resulting CsCl solution was diluted 1 in 4 with TE buffer and the DNA precipitated with ethanol. The concentration of plasmid DNA was assessed from gels against a known concentration standard.

Birnboim-Doly DNA purififcation: This method was modified by Chris Boyd in this laboratory. 100ml cultures of the plasmid containing cells were pelleted by centrifugation at 10K (12000g) for 5mins at 4°C. The pellet was resuspended in 4ml of lysis solution (Birnboim Doly I) and incubated for 5mins on ice. 8ml of of alkaline-SDS solution (Birnboim Doly II) were mixed in and the lysate left on ice for 5mins. 6ml of cold 5M acetate (Birnboim Doly III) was added and mixed by inversion; incubation on ice was continued for a further 10mins. The viscosity decreases sharply as a white floccular precipitate is formed. This was removed by centrifugation at 18K (39200g) for 30mins at 20°C. The plasmid DNA was then precipated at room temperature with 12ml of isopropanol. The DNA was pelleted by spinning at 18K (39200g), 20°C for 30mins. After decanting the supernatant the pellet was carefully washed in cold 70% ethanol. The DNA was

further purified by banding on a CsCl/EtBr gradient. The pellet was resuspended in 2.09ml of TE to which 4.324ml of TE containing 5g of CsCl and 270ul 15mg/ml EtBr was added and mixed. The solution was transferred to a polypropolene 10ml ultra-centrifuge tube, the extra volume being made up with paraffin oil. The gradients were centrifuged in a Beckman Ti70 fixed angle rotor at 49K (200000g) 15°C for 16 hours. The plasmid DNA was purified as above.

Mini DNA Boiling Preparation: Restrictable mini preparations were obtained by the boiling preparation method of Holmes and Quigley (1981). 1.5ml of an overnight culture culture was harvested by centrifugation in a microcentrifuge (12000g) for 30sec. This pellet was resuspended in 350ul of STET buffer and to this was added 25ul of fresh lysozyme (10mg/ml in STET buffer). This was left at room temperature for 5mins and then vortexed briefly. Then the preparations were boiled for 40secs to assist lysis and immediately spun in the microcentrifuge (12000g) at 4°C for 15mins to get rid of debris. The resulting pellet was removed with a toothpick. To precipitate the plasmid DNA 40ul of 3M Na acetate and 400ul of cold isopropanol was added and this was spun down in the microcentrifuge (12000g) at 4°C for 7mins. The supernatant was discarded and the pellet was washed twice with 70% ethanol. The pellet was then dried briefly under vacuum and resuspended in 50ul of TE. RNAase treatment was required prior to loading DNA restrictions onto gels.

Chromosomal DNA Preparations: 100ml cultures of stationary phase cells were harvested at 10K (12000g) for 5mins at 4°C. These cells were resuspended in 3.3ml of 25% sucrose 0.25M Tris pH 8.0 and 0.67ml of lysozyme at a concentration of 5mg/ml(in 0.25M Tris pH 8.0) was added. This was left on ice for 5mins and then 1.3ml of 0.25M EDTA pH8.0 was added to inhibit nucleases. Then an

equal volume of 2% sarkosyl (made up in 50mM Tris 50mM EDTA) was added and this was left on ice for 5mins. This lysate was incubated at room temperature with 0.5ml of RNase at a concentration of 50ug/Ml. Then the lysate was treated with proteinase K (50ug/ml) for 30mins at 37°C and then deproteinized by four phenol extactions, followed by two chloroform extractions to get rid of trace phenol. Great care was taken during these extractions not to shear the chromosomal DNA with rough treatment. The chromosomal DNA was precipitated by adding 1/9 volume of 3M Na acetate and 0.6 volume of isopropanol. This was left at room temperature for 1 hour and then spun down for 25mins at 18K (39200g). The supernatant was discarded and the pellet was washed twice in 70% ethanol. The DNA was then dried and resuspended in 350ul of TE and left to dissolve overnight.

Ethanol Precipitation of DNA: In many experimental procedures DNA must be treated serially with different enzymes requiring different buffers. The most convenient method of changing buffer is to ethanol precipitate the DNA from solution and resuspend the DNA pellet in the second. If required, proteins were removed by phenol extraction and then traces of phenol removed by chloroform extraction before ethanol precipitation. To the DNA to be precipitated 1/9 volume of Na acetate pH 5.3 and 2.5 volumes of ethanol were added. Precipitation was carried out at -20°C for 30mins to 24 hours. The DNA was pelleted by centrifugation at 12000g in a microcentrifuge at 4°C for 30mins. After decanting the supernatant, traces of preipitated salt were washed away by a cold 70% ethanol wash. Finally, the pellet was dried down under vacuum.

Single Colony Gels: This technique enables the plasmid content of an isolate to be observed without the need to isolate DNA. A

single colony was patched out (1cm square) on a selective plate and grown overnight. Using a toothpick a large scrape of cells was collected and resuspended in 200ul of single colony buffer containing 5ul RNase (1mg/ml in TE buffer). The cells were left to lyse at room temperature for 15mins. Cell debris and chromosomal DNA were separated by centrifugation in a microcentrifuge for 15mins (12000g). 50ul of the supernatant containing the plasmid DNA was loaded on an agarose gel. Single colony samples were routinely run on 0.8% agarose gels.

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

Ligation: T4 DNA ligase catalyses the formation of covalently joined hybrid DNA molecules from "sticky" (complementary) ended single stranded DNA molecules to "blunt" ended (no single stranded extension) precursors. Ligation of both sorts were carried out in this work. The plasmid cloning vectors was first digested with the desired restriction enzyme and mixed with compatible restriction fragments of the target DNA. The concentration of fragment to target DNA was adjusted to approximately 3 to 1 for sticky and 10 to 1 molar excess for

blunt ended conings. Blunt ended clonings require a higher molar excess of fragment and a longer incubation period of approximately 16 hours at 16°C. Sticky ended clonings could be effected in 4 hours at 16°C. A typical 20ul reaction mix comprised about 50ng of DNA (vector and fragment), 2ul of 4mM ATP, 2ul of 10x ligation buffer and 0.01 for sticky or 0.1 units for blunt ends of T4 DNA ligase. The volume was made up to 20ul in TE buffer. For blunt ended clonings the reaction volume was reduced to 10ul, all components except the amount of DNA were adjusted accordingly.

Phosphatase Treatment: Self ligation of the cloning vector reduces cloning efficiency dramatically. By removing the 5' terminal phosphate groups on linearized vector prior to mixing with target DNA, self ligation can be minimzed as T4 DNA ligase requires a 5' terminal phosphate on one precursor molecule. Calf intestinal phosphatase (CIP) operates in high, medium and low salt restriction buffers; about 0.01 units were added to vector DNA restriction digest and incubated at 37°C for 30mins. The CIP was heat inactivated at 68°C for 20mins and then the enzymes were removed by phenol extraction.

Conversion of Fragments with Protruding 5' Ends to Blunt Ends: Protruding 5' ends are filled in using the DNA polymerizing activity of the Klenow fragment of E.coli DNA polymerase I. The reaction was set up as follows:

restriction fragment (up to 1ug of DNA in 10ul)	
2mM solution of all four dNTPs	1ul
10x nick-translation buffer	2.5ul
water	to make vol. up to 25ul

To this reaction mix was added 2 units of Klenow fragment of DNA polymerase I. This was mixed and incubated for 30mins at 22°C. Subsequently the Klenow was heat inactivated at 70°C for 5mins.

The blunt ended fragment was then ready for use.

Nick-translation of DNA using ^{32}P

E.coli DNA polymerase I has a 5' to 3' polymerase and a 3' to 5' and 5' to 3' exonucleolytic ability. Therefore, in addition to the polymerisation of nucleotides onto a 3' terminal hydroxyl group, it can also remove nucleotides 5' of a nick and resysnthesise the chain. By replacing a proportion of the free nucleoside bases bases with [a-^{32}P] ATP, it is possible to prepare radiolabelled DNA fagments with a high specific activity. The following were added in order to a small eppendorf tube and constitute a typical reaction mix:

10x Nick-translation buffer	5ul
DNA	0.5ug
unlabelled dNTPs (1mM)	1ul
distilled water	to 40ul

The mixture was chilled on ice, and 0.5ul of DNAase added (0.1ug/ml in nick-translation buffer containing 50% glycerol, stored at -20°C). 5 units of E.coli DNA polymerase I was added and the solution mixed by vortexing. 10uCi [a-^{32}P] dATP (1000Ci/Mole) was mixed in and the solution incubated at 16°C for 60mins. The reaction was stopped by the addition of 2ul of 0.5M Na_2EDTA.

Separation of Nick-translated DNA form unincorparated dNTPs: The nick-translated DNA was separated from unincorparated dNTPs using a column of Sephadex G-50 (medium). A 10ml pipette was rinsed with "replicote" and allowed to dry. The tip was blocked with glass wool, which had previously been soaked in "replicote" and allowed to dry. The column was filled with sephadex G-50 in TE, pH 8.0 and run through with about 3ml of TE buffer, pH 8.0. The nick-translation mixture was added to the column together

with two marker dyes - phenol red, which runs with the unincorparated dNTPs, and dextran blue, which runs with the nick-translated DNA - and eluted with TE buffer, pH 8.0. About 1.5ml of "probe" was collected from the column. These conditions usually resulted in incorparation of between 50% and 70% of the [a-^{32}P]dNTPs into the DNA. The proportion of incorparation was occasionally determined by TCA precipitation assays but this was not done routinely. 0.5ml of nick-translated probe was usually used per hybridization.

Primary Extension Reaction to make Hot cer Probe: Initially the primer was hybridized to the mp18cer in a reaction mix mp18cer 0.5ug DNA, primer 1ul, 10x buffer (100mM Tris pH8.0, 50mM $MgCl_2$) 1.5ul, and distilled water to make reaction mix up to 10ul. This was hybridized at 65^{o}C for 1hour. Then the other strand was synthesised by taking the hybridized template mix 10ul and adding 1ul TTP(100mM), 1ul CTP(100mM), 1ul GTP(100mM), 50uCi[a-^{32}P] dATP (1000ci/mMole) and 1 unit of klenow. This reaction was incubated at 30^{o}C for 60mins. Then the cer fragment was cut out of the mp18 in the following reaction: to the synthesis reaction was added 2ul of 500mM NaCl, 5 units of EcoRI and 5 units of HindIII. This reaction was incubated at 37^{o}C for 1 hour and subsequently run on a 4% acrylamide gel to separate mp18 from the cer fragment.

DNA Transfer to Nylon Membranes and Detection:

(i) Transfer procedure: Southern transfer was performed by a modification of the procedure of Southern (1979). After electrophoresis the gel was removed from the apparatus, stained and photographed, then placed in 150ml of 0.25M HCl for 25mins with gentle agitation. The gel was then rinsed briefly in distilled water prior to gentle agitation with 150ml of

denaturing solution for 30mins. After rinsing again, the gel was agitated for a further 30-60mins in 150ml neutralizing solution. The Southern transfer apparatus was then prepared by placing two reservoirs containing 20xSSC were placed side by side, spanned by a glass plate. A sheet of Whatmann 3MM filter paper was placed over the glass plate from one reservoir to the other and any air bubbles were expelled by rolling a glass pipette over. The gel was placed on the paper/glass support and flanked by strips of sellotape to ensure that the blotting buffer went through the gel rather than around it. A piece of Pall nylon membrane was placed on top of the gel surface and a clean pipette rolled over the membrane to remove trapped air bubbles. Two layers of filter paper were placed on top of the membrane followed by two Boots disposable nappy pads with the outer covers removed. This was covered by a glass plate and a weight of about 1kg. Transfer was allowed to procede overnight (16 hours) after which the membrane was removed from the gel surface, air dried and baked at 80°C for 1 hour. The gel was then restained to check that all the DNA had transferred.

(ii) Prehybridization: The membrane was placed in a plastic bag with 10ml of prehybridization solution (5x Denhardts solution, 5x SSPE, 0.2% SDS). 1mg of sonicated salmon sperm DNA was denatured by the addition of 1/10 volume of 1N NaOH and heating at 65°C for 10mins, then neutralizing by the addition of 1/10 volume of 1M HCl. This was added to the prehybridization solution. The bag was then heat sealed and placed inside a second bag with a wet paper towel. The bags were incubated in a 65°C water bath for 1-2 hours with gentle agitation.

(iii) Hybridization: The bags were opened and the mixture replaced with fresh hybridization solution (as for prehybridization). 0.5ml of nick-translated probe was added to

the salmon sperm DNA and was denatured and neutralized as above, then immediately added to the hybridization solution. The double bag was resealed and incubated at 65°C overnight (16 hours) with gentle agitation.

(iv) Washing Membranes: After hybridization the membrane was rinsed briefly in low stringency wash buffer, then sealed in a plastic bag with about 250ml of the same buffer and agitated for 30mins. This was repeated if only a low stringency wash was required.

(v) Autoradiography:The membrane was exposed to Kodak X-Omat film in a light-proof cassette at -20°C using a single Dupont Cronex lightning-plus intensifying screen. The film was sandwiched between the membrane and the intensifying screen.

2.12 DNA Electrophoresis Through Gels

Unless otherwise stated two gel matrices were used in this work, for DNA of 1kb and above, agarose gels were employed, 0.8% for restriction digests and 0.8% for single colony gels.

Agarose Gels:

(i) Horizontal gels: Agarose powder (geling temperature of 36-42°C) was dissolved at 100°C in 100ml of 1x E buffer in a flask. The molten agarose was then cooled to 55°C prior to use. Perspex or glass gel formers were used along with teflon well formers located at one end of the former. The gel was poured and allowed to solidify. Once the wells were wetted with E buffer the comb was removed. The gel was then removed from the former and placed in a horizontal gel running apparatus. This comprised of two 500ml buffer tanks separated by a raised platform on which the gel was placed. Sufficient 1xE buffer was supplied to just cover

the gel. The samples were loaded using a 5-50ul Finn pipette. Gels were usually run at 5 Volts/cm giving a running time of around 3 hours.

(ii) Vertical Gels: Gel kits which held 16x15cm glass plates separated by 3mm spacers were used. 100ml of molten agar (0.8%) pre-cooled to 55°C was poured between the glass plates after these had been sealed with 10ml of agarose and a 10 or 15 space teflon comb was positioned at the top of the gel. When the gel had set, 1x E buffer was added to the top and bottom reservoirs and the comb removed. A typical single colony gel was run at a maximum of 6.6Volts/cm for around about 3-4 hours. Gels were stained in EtBr (0.05ug/ml) for 30mins prior to being photographed on a 260nm UV transilluminator using a polaroid camera loaded with Polaroid 4x5 land film (no. 57) or a pentax 35mm SLR loaded camera loaded with Ilford HP5 film; both were fitted with a Kodak Wratten Filter No.9 (red).
The interpretation of gel tracks of untreated plasmid DNA were based on Dugaiczyk et al (1975). The fastest migrating band was the supercoiled plasmid monomeric DNA, which is normally the most abundant band. Behind this ran an open circlular plasmid (DNA) band and often comigrating supercoiled plasmid dimeric DNA (2^m). Open circlular dimers and higher forms if visible ran even more slowly. Plasmid linears ran between the supercoiled and open circular plasmid forms. In single colony gels, sheared fragments of chromosomal DNA ran as a single thick band towards the top of the gel.

Sizing of restriction fragments: The distance migrated by a DNA molecule is related to its size such that small molecules migrate the furthest. The mobility of a standard set of molecular weight markers is plotted against the log of the molecular weights of those same markers. From this graph one can calculate the

molecular size of a fragment relative to the set of standards.

Polyacrylamide gels: the gel was polymerized in a vertical gel kit between two glass plates 15x16cm separated by 1.5mm spacers, using a teflon comb to form the wells. The gel apparatus was sealed with 1xTBE 1% agarose. The stock solution of acrylamide was 20% Acrylamide/1% bis (w/v)

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

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

2.13 Extraction of DNA From Gels

Electroelution from agarose gels: After staining, the gel was placed on a long-wave transilluminator (300-360nm) and the band of interest was excised. The gel slice was sealed in a short length of dialysis tubing along with 100-500ul of TE buffer depending upon the size of the slice. The dialysis tubing was placed in a horizontal gel kit and secured using plasticene prior to the addition of 1x E buffer. The slice was electrophoresed at 50V for 4 hours. The current was then reversed for 30sec to remove DNA from the sides of the tubing. The dialysis bag was then opened, the TE removed and the DNA precipitated with ethanol. The DNA was then ready to be used for cloning.

Low Melting Point Agarose: The band of interest was excised from a low melting poit gel, melted at 65°C diluted and mixed with 2

volumes of E-buffer also at 65°C. The mixture was then cooled to 37°C and an equal volume of phenol (room temp) added and vortexed vigorously. The mixture was spun at 12000g in a microcentrifuge for 4mins to separate the aqueous and phenol phases. A white layer is formed at the interface and contains the agarose. Care must be taken not to remove this along with the supernatant since ions in the agarose will interfere with ligation. The supernatant was chloroform extracted 2x and the DNA precipitated with ethanol.

Acrylamide Gels: The DNA was electroluted as above using 1x TBE buffer instead of 1x E buffer. Alternatively for bands from radioactive gels a different elution technique was used which requires less handling of the sample. The band was chopped up into pieces in a 1ml eppendorf tube containing 250ul of elution buffer (500mM NH_4 Acetate, 1mM EDTA) and left overnight at 42°C. The next day this sample was filtered through sand to remove any acrylamide fragments and then phenol extracted, chloroform extracted and precipitated with ethanol.

2.14 SDS-Polyacrylamide Gels for Analysis of Protein Samples

The gel system used was a two part SDS-polyacrylamide gel, a modification of that of Laemmli (1970). The upper part was a short stacking gel (acrylamide concentration = 4.5%) and the lower part was a longer separating gel (acrylamide concentration as stated in the text). The gel was poured in the same apparatus as used for standard polyacrylamide electrophoresis of DNA samples and was poured in two parts:

(i) Separating gel(18%): 12ml of 4x lower buffer was mixed with 4ml of distilled water, 24ml of 30% acrylamide/0.8%NN'bis-methylene acrylamide, 10ul of TEMED and 150ul of 10% APS. This mix was poured between the gel plates to a level 1cm below the

bottom edges of the teeth of the comb. Then isopropanol was poured over the top of the gel to exclude the air and allow polymerization to occur.

(ii) Stacking gel: 2.5ml of upper buffer was mixed with 6.0ml of distilled water, 1.5ml 30% acrylamide/0.8%NN'bis-methylene acrylamide, 20ul of TEMED and 30ul of 10% APS. The isopropanol was then poured of the bottom gel and the surface washed twice with distilled water. The stacking gel was then poured on top of the separating gel, ensuring that no air bubbles formed at the interphase and the comb was inserted. The gel was allowed to polymerise and the comb removed. The gel was placed into the running apparatus, the reservoirs filled with protein gel Tris/glycine running buffer. The wells were then washed out using buffer to remove any unpolymerized acrylamide and the samples loaded. The gel was run at a constant current of 10mA overnight, until the blue dye reached the bottom. The gel plates were then carefully separated and the gel fixed/stained in a solution of 0.1% coomassie brilliant blue, 50% methanol, 10% acetone at 37^{o}C for 60mins with gentle agitation. The stain was then replaced with a solution of 10% methanol, 10% acetone and the gel was destained for up to 24hours, with at least one change of destain solution.

The molecular weight marker proteins were purchased from Sigma and contained the following:

Protein	Approx. Mol. Wt.(kd)
Albumin, Bovine	66,000
Albumin, egg	45,000
Glyceraldehyde-3-phosphate dehydrogenase	36,000
carbonic anhydrase	29,000
Trypsinogen, bovine	24,000
Trypsin, inhibitor	20,100
Lactalbumin	14,200

Preparation of the Samples: 200ul of a late log phase culture were spun down at 12000g in a microcentrfuge. The supernatant was discarded and to this was added 80ul of protein SDS loading buffer (+ B-mercapto ethanol). This was vortexed hard to make a thick cell slurry and boiled at 100°C for 5-10mins. Then 30ul of this sample was loaded onto an SDS-polyacrylamide gel. The remainder of the sample was stored immediately at -20°C.

CHAPTER 3

IS THE RECOMBINASE INVOLVED IN COLE1 PLASMID STABILITY HOST OR PLASMID ENCODED?

3.1 Introduction

The multicopy, naturally occurring plasmid, ColE1 is maintained stably under normal growth conditions; however the cloning vectors related to it, such as pBR322 (Sutcliffe, 1978), are relatively unstable. Summers and Sherratt (1984), presented evidence that plasmid instability appears to be inversely correlated with plasmid multimerization; factors or conditions decreasing multimerization, increase stability. Wild-type ColE1 is stable as it encodes a determinant, called *cer*, that resides in a fragment of 280bp. *cer* acts as a substrate for a site-specific recombinase, that resolves plasmid multimers to monomers. Such recombination occurs independently of the RecA, RecF and RecE pathways of homologous recombination. The *cer* system is postulated to enhance ColE1 stability by maximising the number of independently segregating units in a dividing cell. The *cer* site maps to a *Hpa*II-*Taq*I fragment (3687-3969); when this 280bp region is subcloned into unstable plasmid vectors, their stability is increased (Sherratt *et al*, 1984).

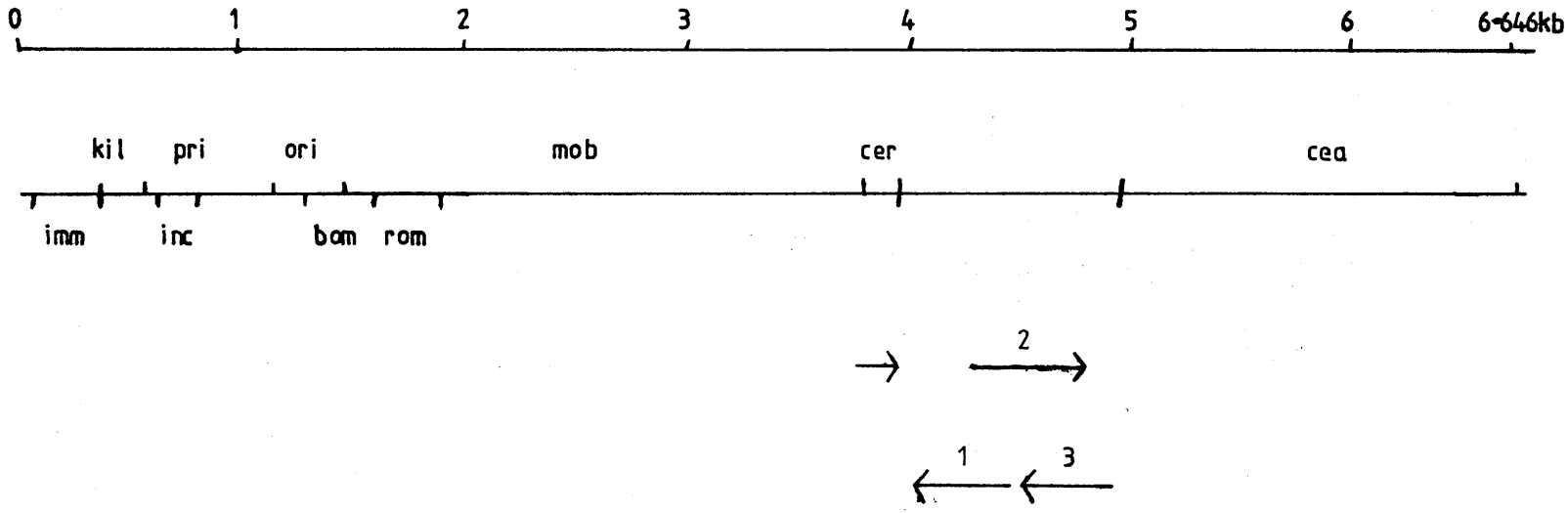


Figure 3.1 General organization of ColE1, showing the cer adjacent open reading frames, 1, 2 and 3.

3.2 Origin of the *cer*-specific recombinase gene.

The recombinase that acts at *cer* was initially thought to be plasmid encoded. In other site-specific recombination systems, such as the Tn3 *res tnpR* system (Arthur and Sherratt, 1979), or the P1 *loxP cre* system (Sternberg and Hamilton, 1981), the gene encoding the recombinase is situated adjacent to the site at which the recombinase acts. Therefore the open reading frames adjacent to the *cer* site in ColE1 were examined for a possible recombinase gene (Figure 3.1). To the left of *cer* lies the *mob* region of ColE1 (2640-3490) and to the other side there there are three open reading frames, ORF1 (4363-3947), ORF2 (4259-4645) and ORF3 (4880-4470). Nauvoma *et al*, (1981) have suggested that ORF 2 is involved in plasmid entry exclusion. The N-terminal of the hypothetical leader polypeptide of ORF2 would contain the leu --- --- cys sequence (Lampen *et al*,1983; Wu *et al*,1983), that is consistent with this polypeptide having a membrane location, which would fit with its role in plasmid entry exclusion. Preliminary experiments using plasmids containing transposon insertions into the *cer* adjacent open reading frames were used to determine whether any of these ORFs are involved in *cer*-specific recombination. The ColE1 Tn1/3 insertion plasmids were examined in a *recBC sbcA* strain, which is hyper-recombinogenic for multicopy plasmids (Fishel *et al*,1981). However disruption of the *cer* adjacent region had no affect upon the ability of these plasmids to break down to monomers, i.e. these transposon insertions had no affect upon *cer*-specific recombination.

In conclusion the recombinase acting at *cer*, must be encoded elsewhere on ColE1, or chromosomally encoded. Alternatively there may be both a host and a plasmid encoded recombinase. The presence of a chromosomal recombinase, would obscure the presence of any homologous plasmid encoded recombinase. Many site-specific recombination systems require ancillary factors in addition to

the recombinase, for example the G inversion system of Mu, which requires a host factor FIS and enhancer sequences (Kahmann et al, 1985). Therefore the cer-site specific recombination system may utilize other factors and/or enhancer sequences and these may be supplied by ColE1 or the host.

Sherratt et al (1984), presented evidence that the only ColE1 sequences required for cer-specific recombination was a region of 280bp containing the cer site. If this 280bp is subcloned into pUC8 or 9, then the plasmid multimers are efficiently monomerized, even in a recBC sbcA strain, in the absence of any complementing plasmid containing ColE1 sequences (Sherratt et al, 1984). There are three possible explanations for these observations:

(i) the 280bp contains a site and a recombinase gene;

(ii) the fragment contains a site, which is acted on by a chromosomally encoded site-specific recombination enzyme, such as the pin gene of E.coli, which acts at the gin, hin and cin inverted repeats (Plasterk, 1983) or

(iii) the plasmid contains a "hot spot" for a chromosomally-encoded general recombination function, having the capacity to monomerize plasmids containing the site. (i) seems unlikely as the region contains an open reading frame, which could code for a polypeptide of 53 amino acids. This putative polypeptide is approximately one third of the size of known recombinases, such as resolvase of Tn3 (Arthur and Sherratt, 1979). The possibility that the cer site may code for some control element of this site-specific recombination system cannot be eliminated. (iii) also seemed unlikely as cer-specific recombination occurs in a wide range of strains, mutant in the RecA, RecF and RecE recombination pathways. Therefore, the second option that cer is acted upon by a chromosomal function seems most likely. This hypothesis was tested, by looking at a range of E.coli strains , to see if there were any differences in cer-specific recombination activities.

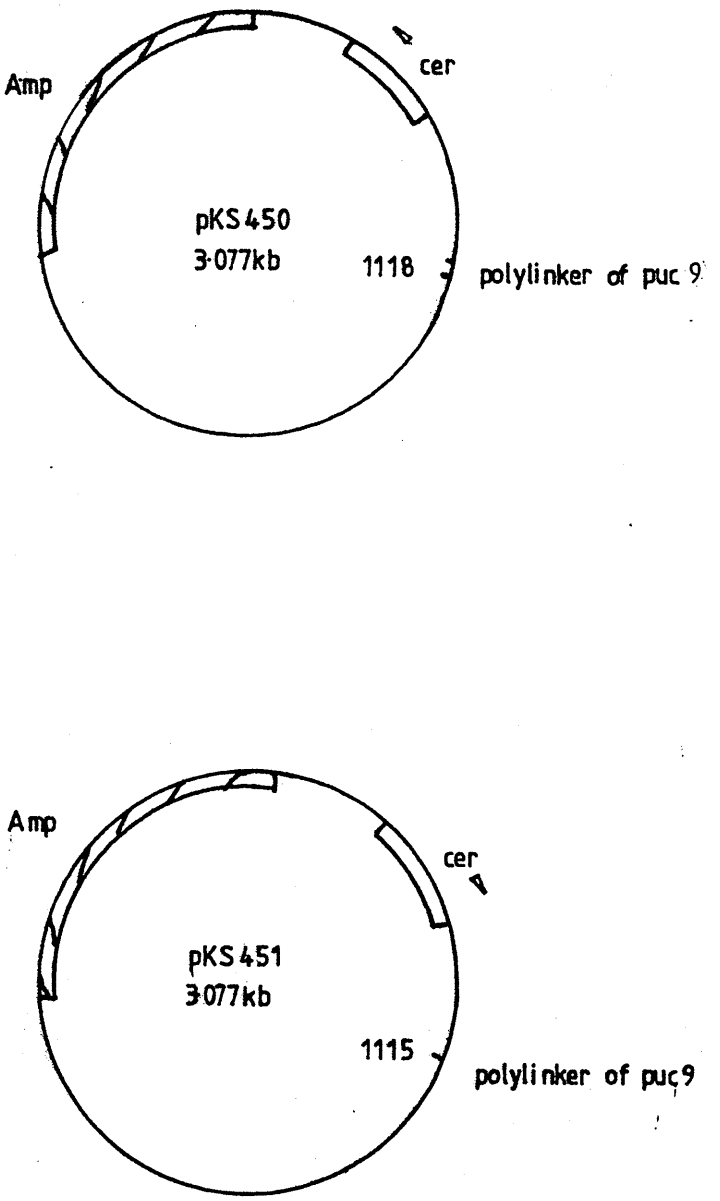


Figure 3.2 Organization of pKS450 and pKS451.

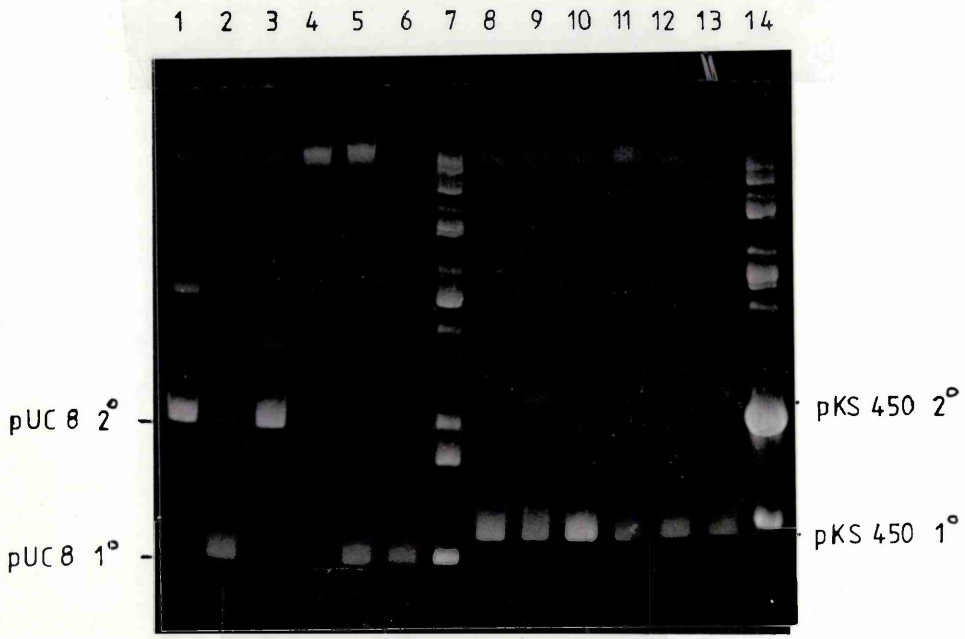


Figure 3.3 The multimerization patterns of pUC8 and pKS450 in a variety of E.coli laboratory strains.
Note the differences in the multimerization patterns of pUC8 and pKS450 in different strains.

Lane (1) pUC8 in DS.801
(2) pUC8 in DS.882
(3) pUC8 in DS.903
(4) pUC8 in JC.8679
(5) pUC8 in DS.803
(6) pUC8 in W5445
(7) pUC8

Lane (8) pKS450 in DS.801
(9) pKS450 in DS.882
(10) pKS450 in DS.903
(11) pKS450 in JC.8679
(12) pKS450 in DS.803
(13) pKS450 in W5445
(14) pKS450

3.3 Strain differences in *cer*-specific recombinase activity.

If there is a host-encoded recombinase, mediating *cer*-specific recombination, then there may be strain differences in its activity or level, particularly if it is not an essential host function. Precedents for this have been shown in other site-specific recombination systems, such as the *fim* system, where the strain HB101, lacks the *fim* genes, controlling the phase switch of type 1 fimbriation in *E.coli* (Klemm, 1986). To determine if such differences occur the *cer*-specific recombination system was examined in various *E.coli* strains.

3.3.1. Analysis of differences in recombinase activity.

In order to investigate this further the ColE1 related plasmids, pUC8/9 (Vieira and Messing, 1982), with or without a *cer* site were transformed into various *E.coli* laboratory strains to see if there were any differences in the multimerization patterns. The plasmids pUC8/9 are normally unstable in *recA*$^+$ strains, tending to form multimers, whereas pUC containing *cer*, pKS450 (Figure 3.2), has increased stability because of decreased multimers (Sherratt *et al*, 1984). The transformants containing pUC8 and pKS450 were examined on single colony gels (Figure 3.3). One of the difficulties of looking at *cer*-specific recombination in this manner is that the levels of host homologous recombination vary from strain to strain. However if the proportion of pUC8 and pKS450 multimers to monomers is compared one can get an indication of the *cer*-specific recombination activities of these strains.

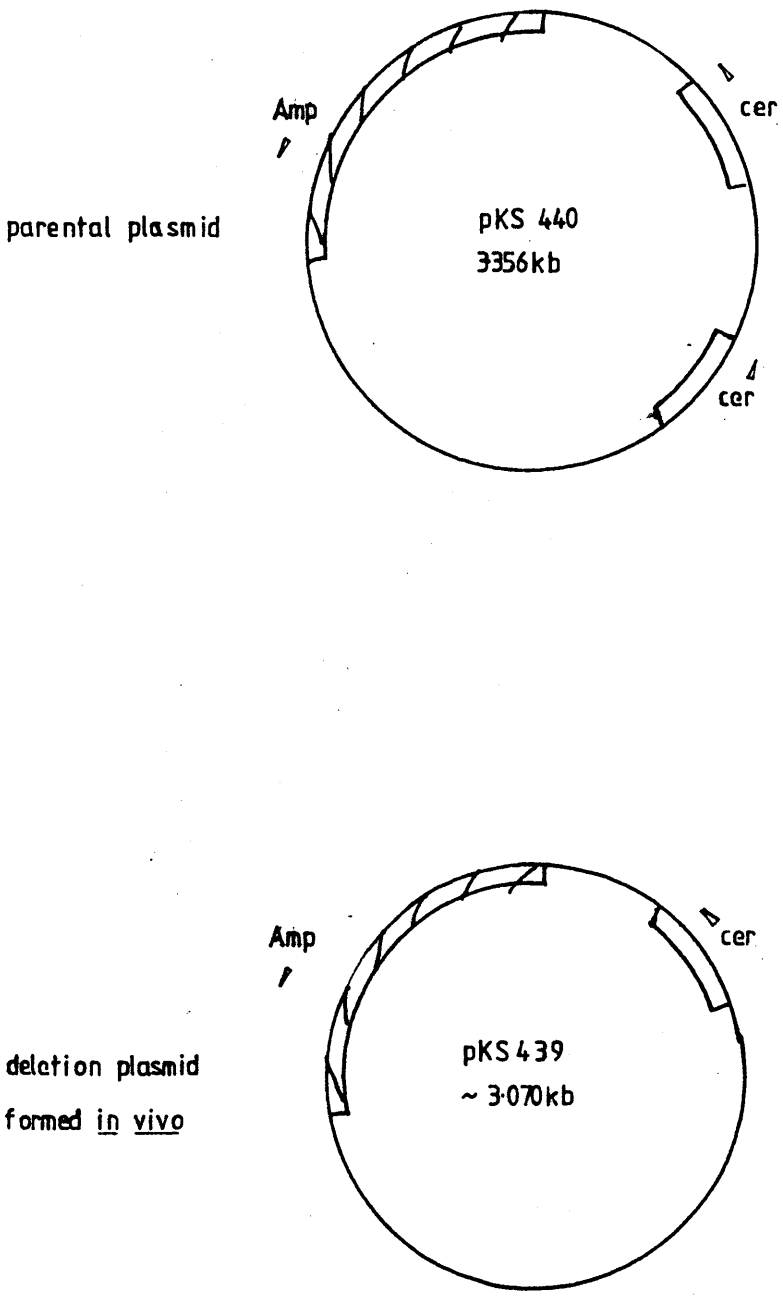


Figure 3.4 The pseudo-cer dimer, pKS440.

This plasmid is derived from pKS450 and contains two cer sites in direct repeat.

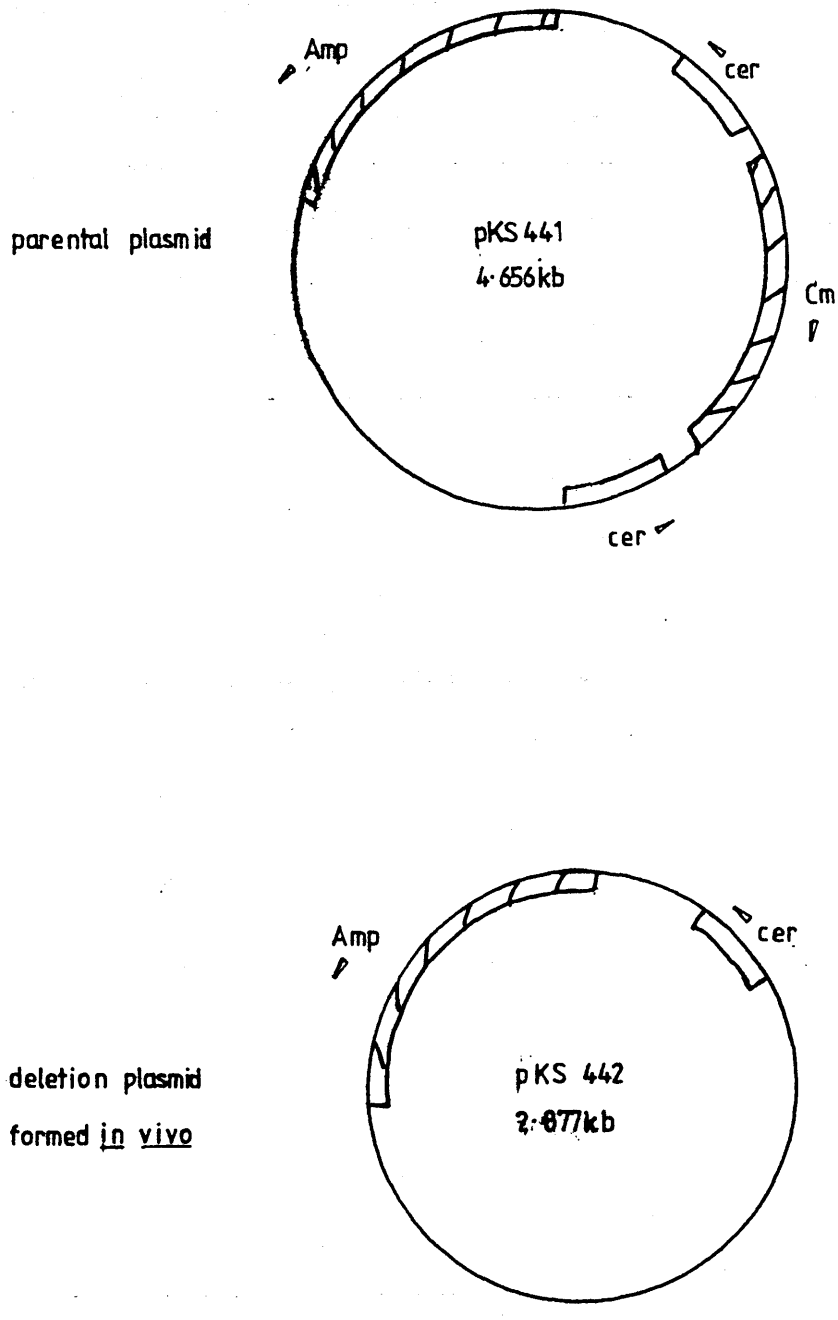


Figure 3.5 The pseudo-cer dimer, pKS441.
This plasmid is derived from pKS440 but contains the gene encoding Cm^r between the two directly repeated cer sites.

3.3.2. Construction of pseudo-*cer* dimers and their behaviour in a variety of *E.coli* laboratory strains.

To confirm these strain differences, a more rigorous assay system than assessing the proportion of monomers to multimers was required. In some strains there may be more plasmidic homologous recombination occurring than in others, thus creating a large proportion of multimers. Multimers are postulated to have a replication advantage over monomers as they contain more origins. If homologous inter-plasmidic recombination is occurring the replication advantage of multimers will lead to the generation of clones of cells containing multimers, which will generate plasmid-free cells at a high frequency (C.T. Jones, 1985). Hence the relative proportion of monomers to multimers will depend on at least three activities: on plasmidic homologous recombination converting monomers to multimers or monomers to multimers, the replication advantage of multimers and on *cer*-specific recombination, resolving multimers to monomers.

David Summers, constructed plasmids containing two *cer* sites in direct repeat. The first of these pseudo-*cer* dimers was pKS440 (Figure 3.4) and the second was pKS441 (Figure 3.5), which is a more useful derivative than pKS440 because it has the chloramphenicol resistance gene inserted between two directly repeated *cer* sites. The latter construct has the advantage of having an antibiotic resistance gene between the two *cer* sites, therefore *cer*-mediated deletion can be followed.

Most *E.coli* K-12 strains appear to have some chromosomal *cer*-recombinase activity and therefore readily form deletion derivatives of the pseudo-*cer* dimers, pKS440 and pKS441, hence obtaining pure parental plasmid was very difficult. The strain W5445 has partial *cer*-specific recombination activity and was used to isolate parental plasmid of pKS440 and pKS441. However W5445 is *recA*$^+$, therefore parental and deletion oligomers of the

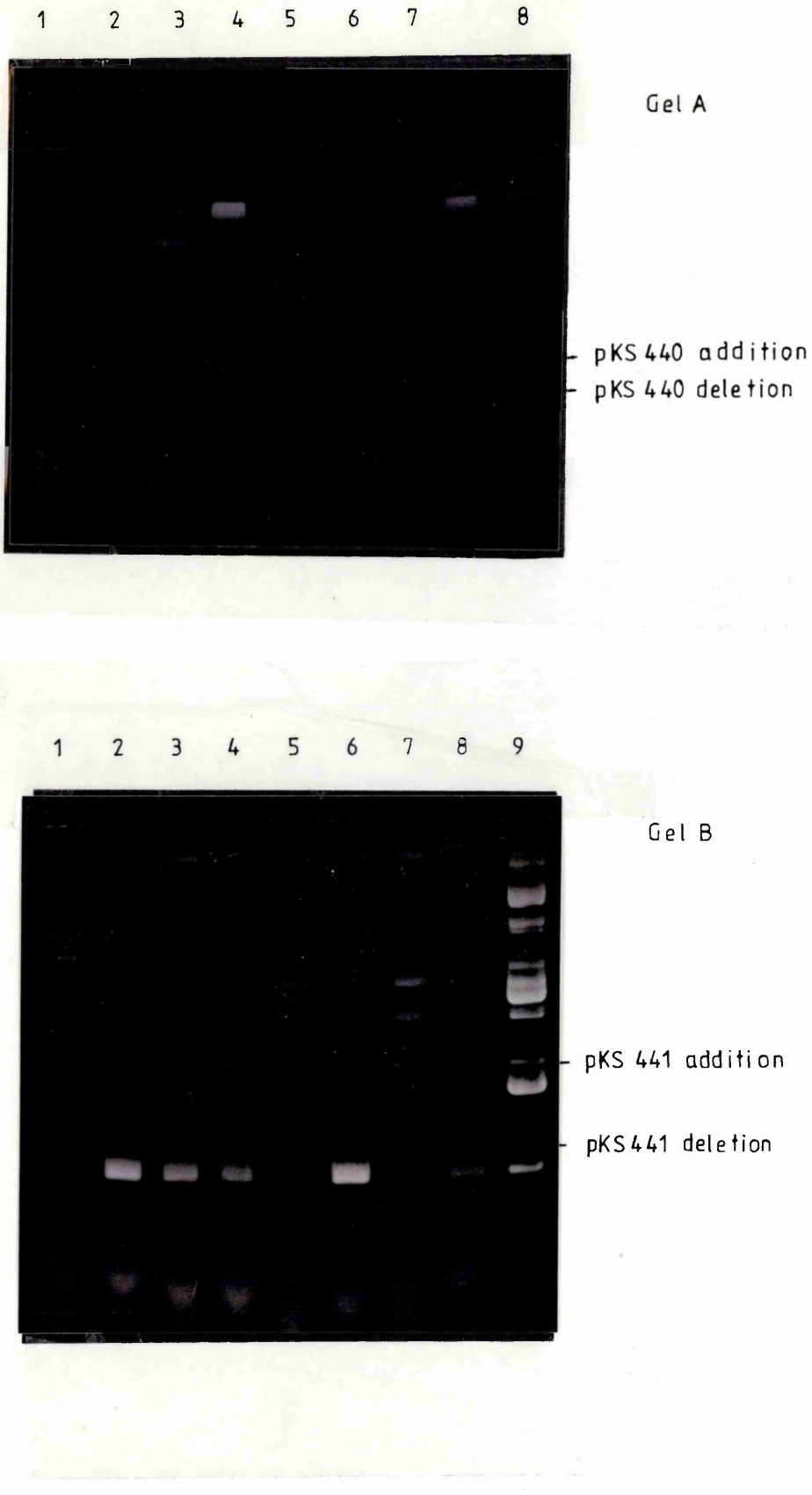


Figure 3.6 Behaviour of the pseudo-cer dimers in a variety of E.coli laboratory strains.

Note the differences in the proportion of parental to deletion forms of the pseudo-cer dimers in different strains.

Single colony gel A

Lane (1) pKS440 in CB1

(2) pKS440 in DS.903

(3) pKS440 in CB.51

(4) pKS440 in CB.50

(5) pKS440 in DS.882

(6) pKS440 in DS.807

(7) pKS440 in W5445

(8) pKS440 in parental form

Single colony gel B

Lane (1) pKS441 in CB1

(2) pKS441 in DS.903

(3) pKS441 in CB.51

(4) pKS441 in CB.50

(5) pKS441 in DS.882

(6) pKS441 in DS.807

(7) pKS441 in W5445

(8) pKS441 in DS.803

(9) pKS441 parental and deletion form

Strain	*cer*-specific recombinase activity
AB1157 CB 1 CB 50 CB51 CR63/1 *E.coli* B	+ +
E.coli C W5445	+/−
DSX.1 DSX.2 DSX.300	−

Figure 3.7 Table of *cer*-specific recombination activities.

This table was constructed from gel analysis of the pseudo-*cer* dimers in a variety of *E.coli* strains.

pseudo-cer dimers were present making isolation of pure parental plasmid more complex. Hence a recA$^-$ derivative of W5445 was constructed by P1 transduction from a strain KL16, which has Tn10, inserted in srl close to the recA13 locus (Kleckner, 1977). The recA status of these Tc^r transductants was tested using UV sensitivity. Thus a recA13 derivative of W5445 was constructed. The ligation mixes of pKS440 and pKS441 were transformed into the strain, W5445 recA13. The colonies which grew were patched out and examined on single colony gels, run in low melting point agarose, with markers of appropriate size for the parental and deletion forms of these plasmids. The parental forms were purified from low melting point agarose and then transformed into a number of different E.coli laboratory strains, and their cer-specific recombinase activities were examined on single colony gels (Figure 3.6). These gels show that the proportion of addition and deletion of the pseudo-cer dimers varies in different E. coli laboratory strains. From examination of these gels, a table of E.coli strains , showing their cer-specific recombinase phenotype can be constructed (Figure 3.7).

The strain W5445 shows differences in the proportion of parental to deletion plasmid, between different transformants. Elaine Berne studied this affect and found that the proportion of parental to deletion forms in different W5445 transformants stayed reasonably constant over a number of repatchings. There are a number of possible explanations for this observation. One explanation for this could be that the cer site produces a repressor of the cer-specific recombinase. So in the transformation of W5445, a cell receives a copy of a pseudo-cer dimer, therefore the amount of repressor is very low, allowing the cer-specific recombinase to act until the level of repressor reaches a certain level. After the initial burst of cer-specific recombination, the proportion of parental to deletion plasmid would remain constant. The only situation in which the

recombinase would act again, would be if in dividing cells, the copy number in any cell decreased due to segregation and so the level of repressor would decrease. This would allow a burst of cer-specific recombination, generating monomers, which maximises the number of segregating plasmid units. The biological significance of such a system for ColE1 could be to regulate cer-specific recombination. In dividing cells, it is believed that a small proportion of cells receive very few copies of ColE1. If this was so it would be advantageous to ColE1 to maximise the number of segregating units by breaking down any multimers present.

3.4 Southern blot analysis of E.coli DNA for homology to ColE1.

The presence of a host encoded recombinase could obscure any homologous ColE1 encoded, cer-specific, recombinase activity. If the plasmid and host encoded recombinases are interchangeable they should be fairly homologous at the DNA level. This homology was screened for using ColE1 itself, various deletions and fragments of it, to probe to a variety of E.coli chromosomal DNAs.

3.4.1 Screening the E.coli chromosome for homology to ColE1 and ColE1 deletion derivatives.

The first screen of the E.coli chromosome for a cer-specific recombinase used as a probe, ColE1. The chromosomal DNA of a number of E.coli strains was digested with EcoRI and run on a 0.8% agarose gel. This was then transferred onto pall membrane by Southern blotting and probed, using nick translated ColE1. The pre-hybidization and hybridizations were carried out at 65°C and a low stringency wash was used as described in Materials and Methods. When the autoradiograph was examined, no homology was

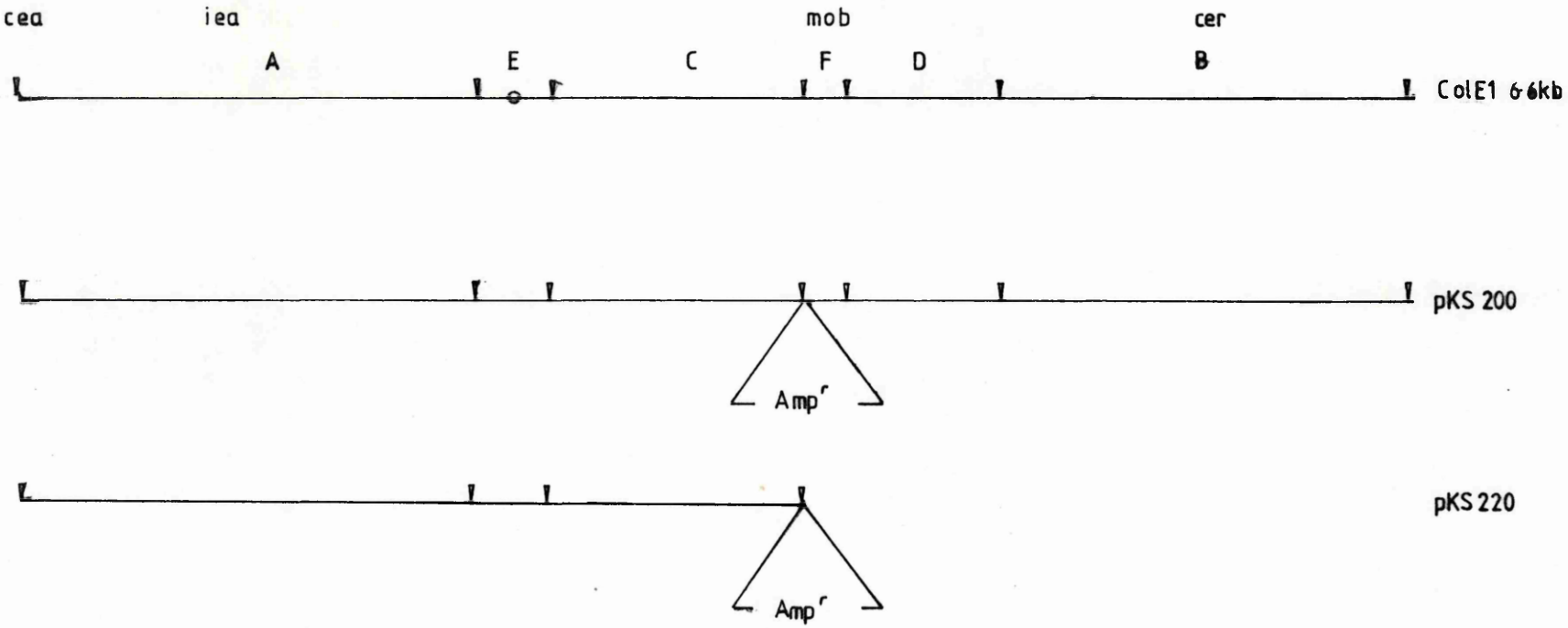


Figure 3.8 HaeII restriction map of Amp^r ColE1 derivatives. The map indicates the position of the HaeII sites ▼ , the replication origin ○ and the position of the HaeII fragment carrying the Amp^r determinant of Tn3 (Summers and Sherratt, 1984).

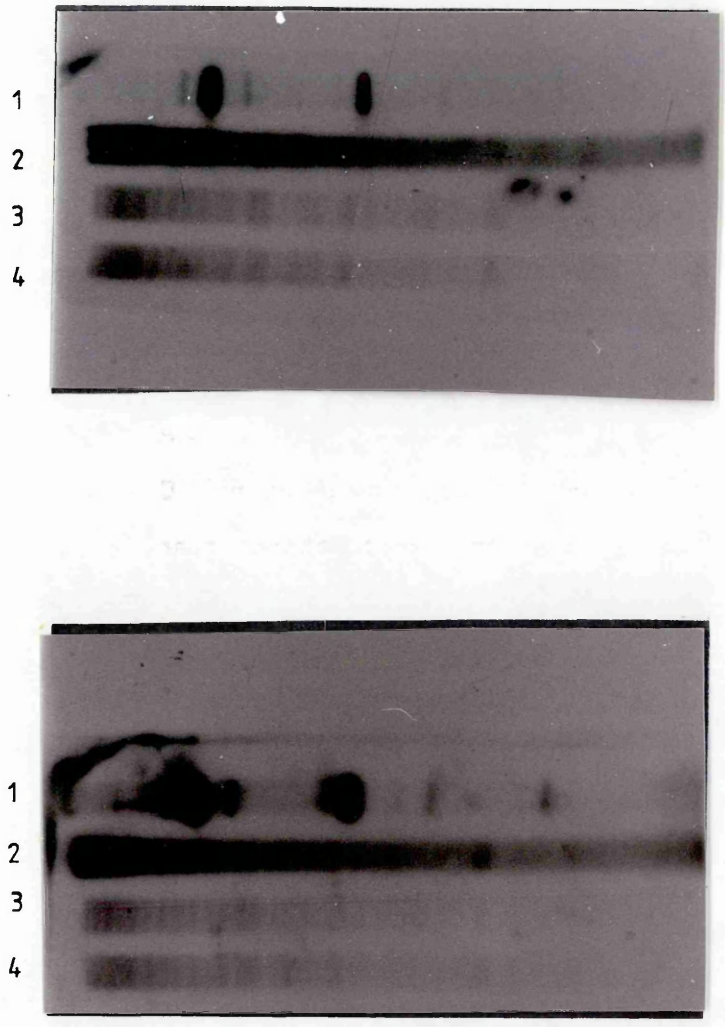


Figure 3.9 Differential probing of E.coli chromosomal DNA using pKS200 and pKS220.

Two identical Southern blots were probed with (A) pKS200

(B) pKS220

Lane (1) ColE1

(2) E.coli C DNA digested with EcoRI

(3) DS.911 DNA digested with EcoRI

(4) DS.903 DNA digested with EcoRI

detected. This was not conclusive, perhaps due to the non-specific nature of the probe.

Then a differential screen was used, probing two identical Southern blots with two ColE1 Amp^r derivatives, pKS200 and pKS220 (Summers and Sherratt, 1984). pKS200 has the HaeIIB fragment containing cer and the adjacent open reading frames 1,2 and 3, whereas pKS220 does not (Figure 3.8). This differential screening was designed to pick out any bands with specific homology to the cer region of ColE1. The pre-hybridization and hybridization were carried out at 65° C and a low stringency wash was used as described in Materials and Methods. No specific bands were picked out by the HaeIIB fragment of ColE1 (Figure 3.9). There were no differences between the two blots.

3.4.2 Screening the E.coli chromosome using fragments of ColE1 as a probe.

The first fragment used to screen E.coli laboratory strains for homology to the cer region of ColE1 was the HaeIIB fragment of ColE1. The HaeIIB fragment was purified from a low melting point agarose gel and then nick translated. Prehybridization and hybridization were carried out at 65°C, and a low stringency wash was used as described in Materials and Methods. No homology to the HaeIIB fragment was detected.

The next fragment used to screen the E.coli chromosome for homology to cer was the 377bp HpaII cer fragment. This 377bp fragment was cloned into M13 and a probe was made using a primary extension reaction. After the primary extension reaction the cer fragment was cut out of M13 using EcoRI and HindIII, then this restriction was run on a 4% acrylamide gel using xylene cyanol as a marker dye. From a 4% acrylamide gel the labelled 377bp fragment was cut out. The 377bp fragment was purified from the gel slice by the elution technique described in Materials and

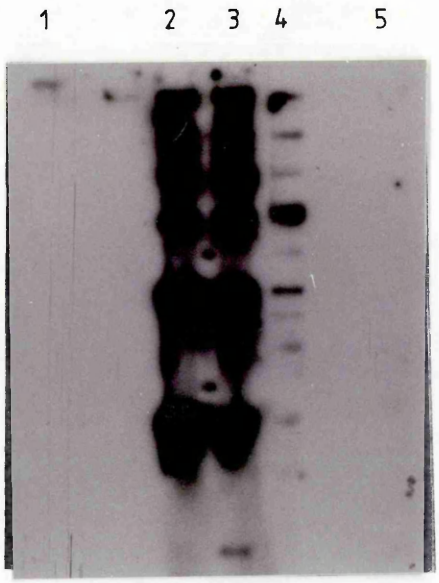


Figure 3.10 Probing *E.coli* chromosomal DNA for homology to the 377bp *HpaII cer* fragment.

Lane (1) DS.903 DNA digested with *EcoRI*

(2) pKS450

(3) pRY10

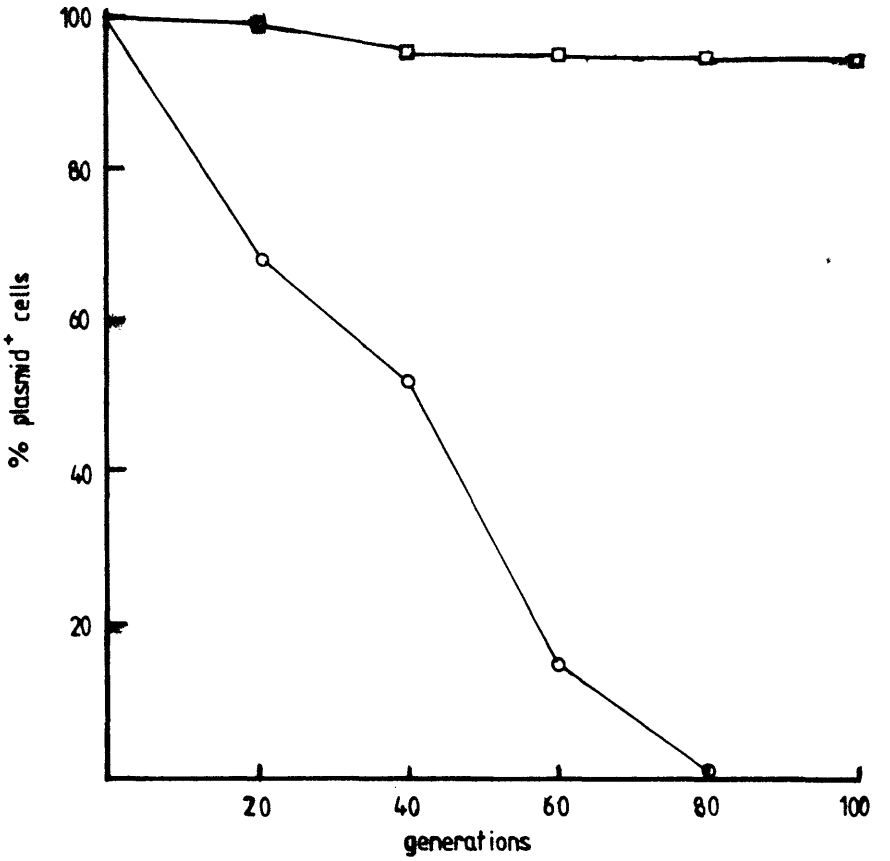
(4) pEV87

(5) DS.903 DNA digested with *EcoRI*

Methods. On the gel to be blotted the following were run: DS.903 chromosomal DNA, digested with EcoRI; pKS450 DNA, containing cer, which has 100% homology to cer; pRY10 DNA, containing ckr, which has 90% homology to cer over a 150bp core region of cer (Summers et al, 1985) and pEV87 DNA, containing crl the analogous site in CloDF13, which shows 69% homology with the core region of cer (Sherratt et al, 1984). These three plasmids were included as they show a spectrum of homology to cer. If ColE1 had for example utilized a recombinase function of a transposon then one might expect the cer site to exhibit some homology to the site in the transposon, however they may have diverged significantly, hence the reason for including in the Southern blot plasmids with a gradient of homology to cer.

When the primary extended cer fragment was used as a probe, homology to cer, ckr and crl was detected, but not to any DS.903 chromosomal bands (Figure 3.10). However this does not mean that ColE1 is not using a chromosomal recombinase to act at cer. If ColE1 was utilizing the recombinase of a transposon, then the cer site and the transposon site may have diverged significantly. Another possibility is that only the actual crossover region is conserved, this has been localized in cer to a 35bp region (Summers et al, 1985). From later work, a wild type chromosomal gene was isolated and characterized. This clone contains a region of 14bp which has very strong homology to the crossover region of cer. The washing conditions used in this experiment - 2xSSC- would have only detected sequences which were 50% homologous to the probe. Therefore it may not be surprising that the ColE1 probes did not detect homology to any site in the E.coli chromosome.

Graph A



Graph B

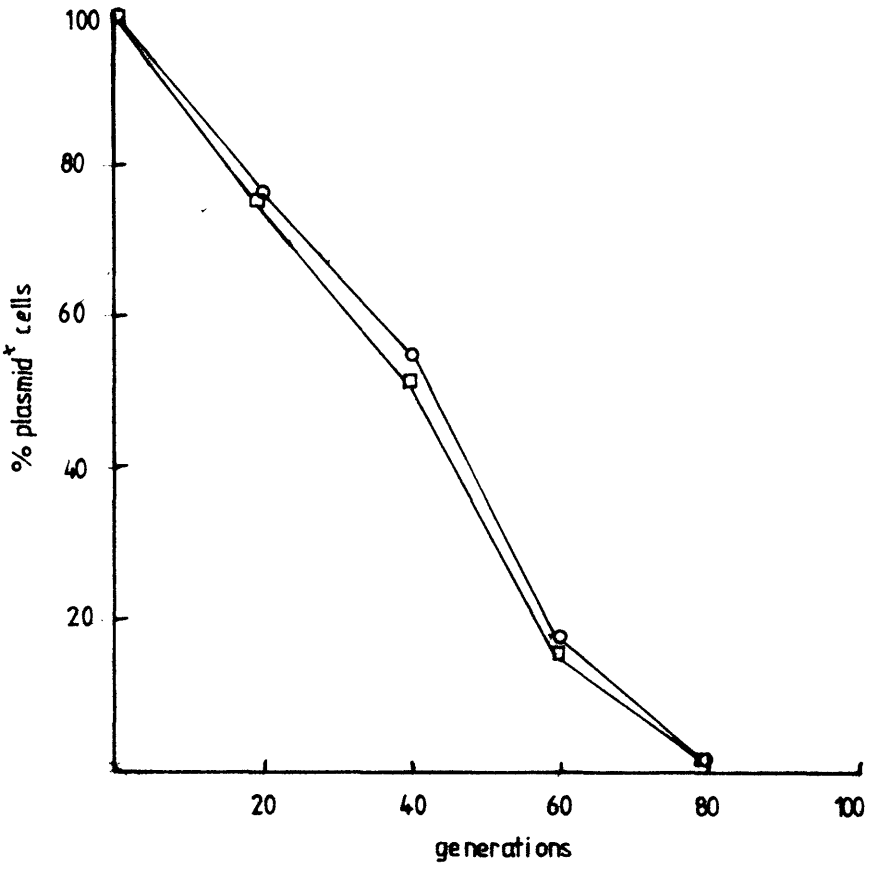


Figure 3.11 Stability of pKS200 and pKS220 in isogenic *xer*$^+$ and *xer*$^-$ hosts, under non-selective growth conditions.

Graph (A) The stability of pKS200 measured in a *xer*$^+$ host (□) or in a *xer*$^-$ host (○).

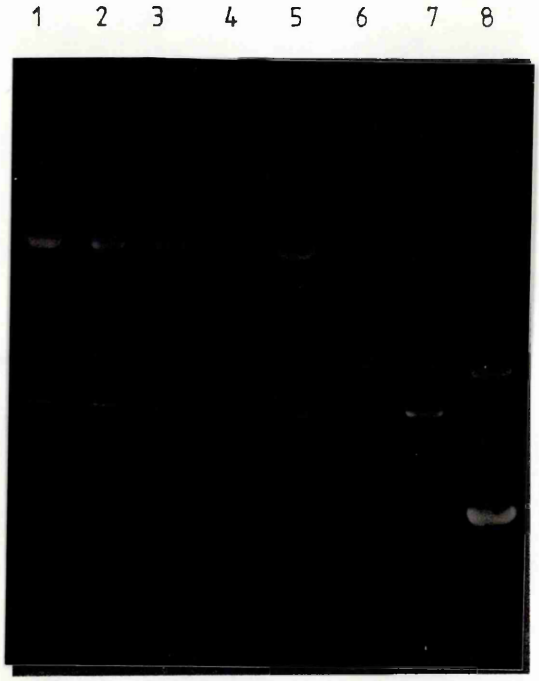
Graph (B) The stability of pKS220 measured in a *xer*$^+$ host (□) or in a *xer*$^-$ host (○).

3.5 Use of *xer* mutants to determine if there is a plasmid recombinase.

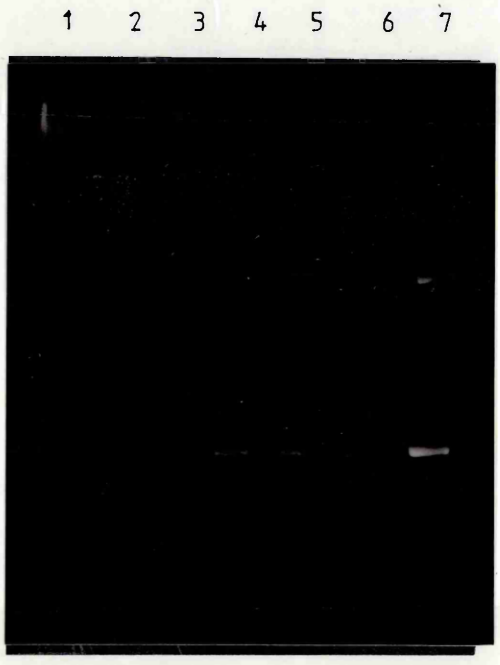
The question of whether or not there is a plasmid encoded recombinase may until now have been obscured by the presence of an analogous host encoded function. Now that host function deficient mutants (*xer*$^-$) have been constructed, it is possible to address this question (derivation of mutants discussed in Chapter 4). The host genes, coding for functions which act at *cer* have been called the *xer* genes.

3.5.1 Stability of Amp^r derivatives of ColE1 in *xer*$^{+/-}$ strains.

The stability of two Amp^r *cer*$^{+/-}$ derivatives of ColE1, pKS200 and pKS220 (Summers and Sherratt, 1984) (Figure 3.8), were examined in *xer*$^{+/-}$ hosts. The plate test of Boyko and Ganshow (1982) would normally have been used to assess the %B-lactamase$^+$ cells, however these ColE1 derivatives have a low copy number in the *xer*$^-$ hosts, which gives false negative results. The stability was therefore assessed by comparing the number of viable cells and the number of Amp^r cells at 20 generation intervals from 0 to 100 generations. These data were plotted as %B-lactamase$^+$ cells versus number of generations (Figure 3.11). It was observed that: pKS220, containing no *cer* site, is unstable in a *xer*$^{+/-}$ host, as expected and that pKS200, containing a *cer* site, is unstable in a *xer*$^-$ host but stable in a *xer*$^+$ host. pKS220 in a *xer*$^{+/-}$ host and pKS200 in a *xer*$^-$ host, follow the same trend of instability. It can be inferred from this that not having a *xer* function, has the same effect upon the stability of an Amp^r ColE1 derivative, as not having a *cer* site. Whereas when the ColE1 Amp^r derivative, has a *cer* site and is in a *xer*$^+$ host, it is as stable as wild type ColE1 in a *xer*$^+$ strain. ColE1 can not supply the



Gel A



Gel B

Figure 3.12 Single colony gel analysis of ColE1 and pACYC184 in isogenic *xer*$^{+}$ and *xer*$^{-}$ hosts.

Gel (A) First patching after transformation.

Lanes (1-3) ColE1 + pACYC184 in CSH.26

(4-6) ColE1 + pACYC184 in DSX.300

(7) ColE1

(8) pACYC184

Gel (B) Isolates from gelA repatched and examined on single colony gel.

Lanes (1-3) ColE1 in DSX.300

(4-6) ColE1 in CSH.26

(7) ColE1

Note that ColE1 has been lost from DSX.300, the *xer*$^-$ derivative of CSH26.

function absent in a *xer*$^-$ strain unless in pKS200 the insertion of the gene encoding Amp^r has disrupted the gene coding for a function which could complement the Xer$^-$ phenotype of the host.

3.5.2 Analysis of the correlation between multimerization and instability for ColE1.

The data regarding the stability of pKS200 in *xer*$^{+/-}$ hosts, shows that an Amp^r *cer*$^+$ derivative of ColE1 is unstable in a *xer*$^-$ host, but is this instability related to an increase in multimerization? Summers and Sherratt (1984), presented data for pACYC184, showing a correlation between multimerization and instability; does this correlation hold for ColE1? To determine this the multimerization of the natural plasmid ColE1 was examined in CSH26 and a *xer*$^-$ derivative, DSX.300. ColE1 was co-transformed into these two strains with pACYC184, initially selecting for Cm^r, and then screening for colicin production by the method described in Materials and Methods. Approximately one fifth of the transformants containing pACYC184 also contained ColE1. The colicin producing Cm^r transformants of CSH26 *xer*$^{+/-}$ were examined on single colony gels (Figure 3.12A). These gels show that in the parental strain CSH26 *xer*$^+$, ColE1 is present mainly in the monomeric form, but in DSX300, the *xer*$^-$ version of CSH26 it is present at a lower copy number and as higher forms. These clones were repatched on L-agar going through single colonies several times, to try and get rid of pACYC184, which is normally unstable and then they were examined again on single colony gels (Figure 3.12B). These gels show ColE1 present in the *xer*$^+$ host, but not in the isogenic *xer*$^-$ strain. These results imply that ColE1 forms multimers in *xer*$^-$ strains and is itself unstable in this strain compared to the parental *xer*$^+$ host, in which ColE1 is stable and mainly monomeric. This confirms that the correlation between multimerization and instability, reported

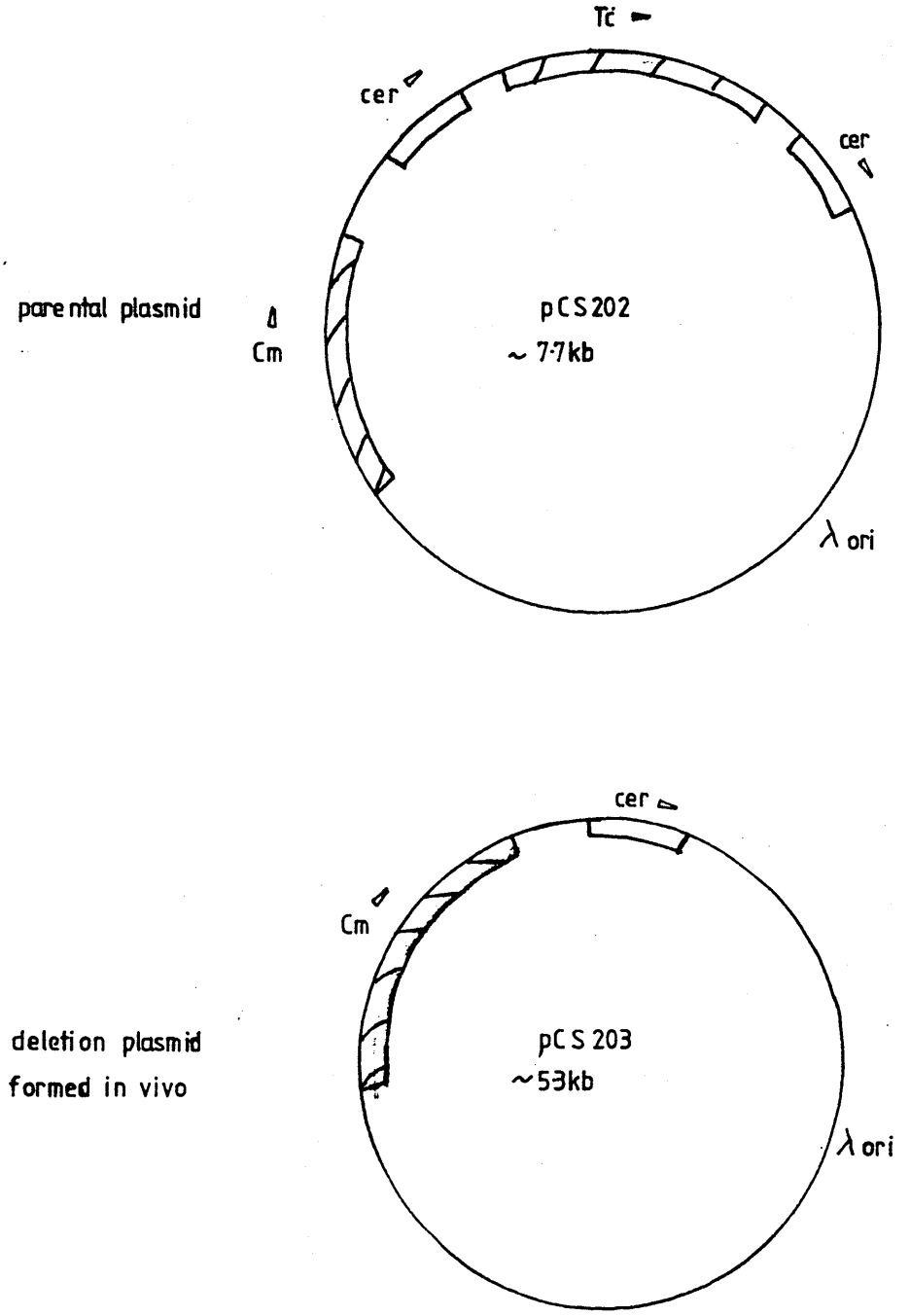
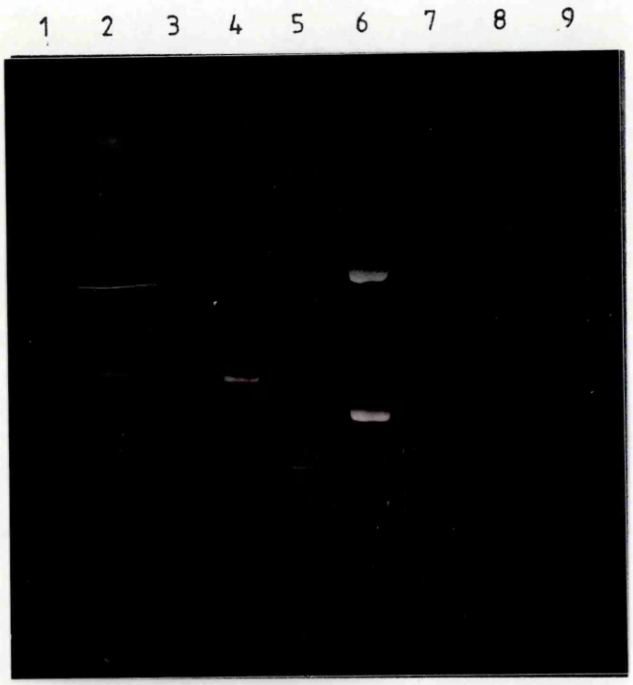
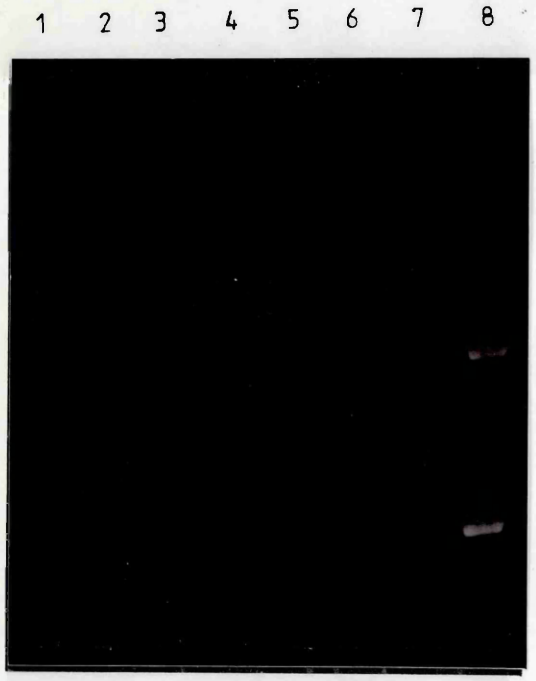


Figure 3.13 Organization of the λ-DV pseudo-cer dimer, pCS202.



Gel A



Gel B

Figure 3.14 The <u>xer</u>⁻ mutants, DSX.1, DSX.2 and DSX.330 containing pCS202 and ColE1.

Note that ColE1 can not supply in <u>trans</u> any of the functions absent in DSX.1, DSX,2 and DSX.330.

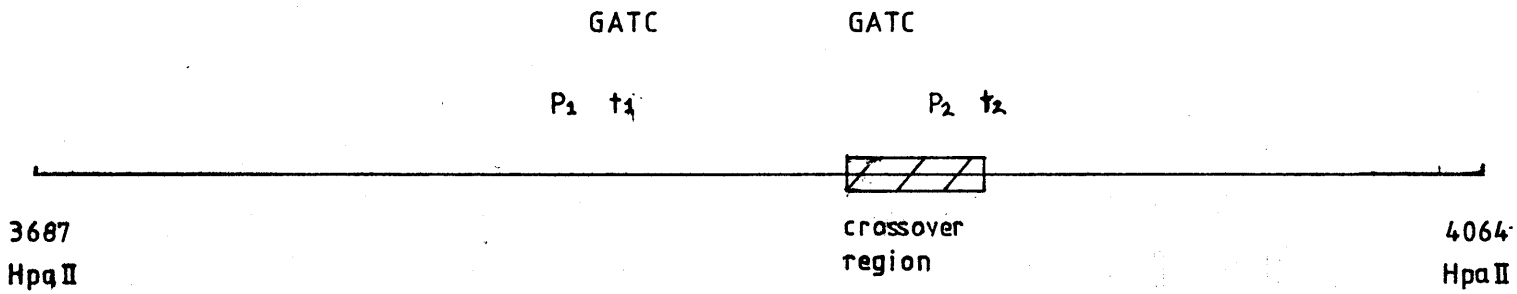


Figure 3.15 The dam methylation sites in cer.

for pACYC184 (Summers and Sherratt, 1984), holds for ColE1 itself.

3.5.3 Can the function, absent in a *xer*⁻ strain acting at *cer*, be supplied in *trans* by ColE1?

To show that the functions absent in the *xer*⁻ mutants, DSX.1, DSX.2 and DSX.330 cannot be supplied in *trans* by ColE1, the three mutants were co-transformed with ColE1 and a pseudo *cer* dimer of DV origin, called pCS202 (Figure 3.13), selecting for pCS202 using chloramphenicol. The Cm^r transformants were screened for colicin production as described in Materials and Methods. Approximately one out of every five cells, which had received pCS202 had also received ColE1. The transformants containing ColE1 and pCS202 for each of the three mutants DSX1, DSX2 and DSX330 were examined on single colony gels (Figure 3.14). These gels show that pCS202 remains in the parental form in each of the three mutants in the presence or absence of ColE1. Implying that ColE1 cannot supply the function or functions which are absent in any of the three *xer*⁻ mutants. These data also suggest that the functions mutant in the three *xer*⁻ strains are not repressing a plasmid encoded function, which can complement the Xer⁻ phenotype.

3.6 The affect of *dam* methylation on *cer*-specific recombination.

Within the *cer* determinant of 280bp lie two *dam* methylation sites, GATC, one at T1 and one before P2 (Figure 3.15). Methylation could be used as a means of timing of *cer*-specific recombinase activity. A hypothesis could postulate that the recombinase would be unable to act on methylated DNA, but that it could act on hemi-methylated DNA: allowing a burst of *cer*-specific recombinase activity just after replication. There are

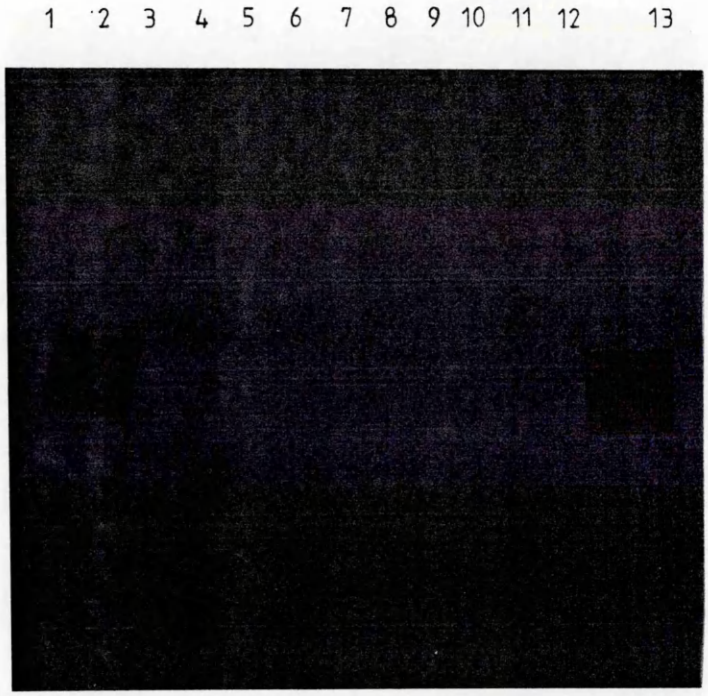


Figure 3.16 Methylated and non-methylated pKS450 transformed into isogenic dam$^+$ and dam$^-$ strains.

Lanes (1-3) pKS450 from dam$^+$ strain in CB.50

(4-6) pKS450 from dam$^+$ strain in CB.51

(7-9) pKS450 from dam$^-$ strain in CB.50

(9-12) pKS450 from dam$^-$ strain in CB.51

(13) pKS450

two dam sites in Tn10, one of which is in the promoter of the transposase gene. Kleckner et al (1984), have shown that Tn10 transposition is increased ten fold in a dam$^-$ strain. They have therefore proposed that dam methylation could be used as a timing mechanism for transposition in the cell cycle.

The initial observation indicating that there may be an affect of dam methylation on cer-specific recombination, was looking at the pseudo-cer dimer, pKS441, in strains isogenic in genotype except for their dam status, one being dam$^+$, CB50 and the other being dam$^-$, CB51. In these two strains the proportion of multimers to monomers was very different, so this was investigated further, as they are isogenic apart from their dam genotype.

DNA of pKS450 was made in CB50 and CB51 and then this methylated and non-methylated DNA was transformed into both CB50 and CB51 selecting on L-agar + Amp. The Amp^r colonies were examined on single colony gels (Figure 3.16). In the dam$^+$ strain, CB50 the DNA made in the strains CB50 and CB51 was the same with respect to multimerization patterns. The same was true in the dam$^-$ strain, CB51 i.e. the multimerization pattern was the same irrespective of the methylation status of the transforming DNA. It would appear that pKS450 or any cer$^+$ plasmid has a higher copy number in a dam$^+$ strain than in a dam$^-$ strain. The original observation with the pseudo-cer dimer, pKS441, was that in CB50 there was a higher proportion of multimers than in CB51. The explanation probably is that in the dam$^+$ strain, CB50, plasmids have a higher copy number hence it is more likely that multimers will be visible in the dam$^+$ strain than in a dam$^-$ strain.

In conclusion dam dependant methylation appears not to have any obvious affect on cer-specific recombination. However if isogenic dam methylation proficient and deficient strains are compared they show many differences in behaviour e.g. differences in copy number of plasmids; differences in transforming

efficiencies and a difference in general fitness. Probably if there was an affect of dam dependent methylation on cer-specific recombinase activity, it would be masked by other differences in behaviour.

To investigate this further it would be necessary to change the dam dependant methylation sites in cer, by site directed mutagenesis and see if this change has any affect on cer-specific recombination.

Discussion and conclusions.

Our initial view of the site-specific recombination system of ColE1 was that there was a site, cer, and a recombinase which acted at that site. The cer site has been localized to a 280bp region of ColE1 (3687-3969) (Sherratt et al, 1984). The recombinase gene was not identified but there are precedents in other site specific reombination systems, such as Tn3 res, tnpR system, where the recombinase gene is situated adjacent to the site at which it acts, leading us to look at the open reading frames adjacent to cer. To the left of cer lie the ORFs of mob (Chan, 1986), but to the right, lie the ORFs of undesignated function ORFs 1,2 and 3. Nauvoma et al, (1981) postulate a role for ORF 2 in plasmid entry exclusion. Disruption of cer adjacent region by transposon insertion, does not affect cer-mediated recombination, which implies that the recombinase acting at cer is encoded elsewhere on ColE1 or chromosomally.

When a 280bp fragment of ColE1 is subcloned into pUC8 and 9 efficient monomerization of plasmid multimers occurs, even in a recBC sbcA strain, with no complementing plasmid present (Sherratt et al, 1984). This can be explained in three ways, but the evidence tends to eliminate two of the possibilities. The first option is that the cer fragment itself encodes the site and the recombinase gene, however this region of 280bp contains only

one ORF, which could encode for a polypeptide of 53 amino acids. This polypeptide is only approximately one third of the size of known recombinases, for example resolvase of Tn3, which is 175 amino acids. The ORF in cer is conserved in ColK, which could imply that it has a function or that the region of the site itself is well conserved, which would in turn conserve the ORF. The second possibility is that the cer fragment contains a recombination "hot spot" for a chromosomally encoded general host recombination function, however cer-mediated recombination occurs in a wide range of strains mutant in recombination pathways, so this seems unlikely. This leaves us with the third and most likely option, that the fragment contains the cer site, which is acted upon by a chromosomal function. Other site-specific recombination systems have host encoded recombinases, such as the pin function of E.coli which acts at the hin, gin and cin inverted repeats (Plasterk, 1983). To investigate this possibility, pseudo cer-dimers were constructed. These plasmids were examined in a variety of E.coli laboratory strains, which showed a spectrum of cer-specific recombinase activities. Strain differences would not be expected to occur, if host factors were not required for cer-specific recombination. To investigate the host component of the cer recombination system, mutants were isolated and characterized (Chapter 4). The ColE1 cer-specific recombination system confers stability by providing a means of breaking down multimers, formed by homologous recombination, to monomers. This system is postulated to enhance ColE1 stability by maximising the number of independently segregating plasmid copies within a dividing cell. Summers and Sherratt (1984), demonstrated a direct relationship between multimerization and instability for pACYC184. The data presented here implies that there is a correlation between multimerization and instability for the natural plasmid ColE1.

The absence of a cer site or a xer encoded function has been

shown to cause the same pattern of instability of ColE1 Amp^r derivatives, implying that both cer and xer are required for ColE1 plasmid stability. The functions absent in the three xer^- mutants, DSX1, DSX2 and DSX330, cannot be supplied in trans by ColE1 itself. In wild type E.coli the presence of a host cer-specific recombinase could have obscured or repressed any ColE1 encoded recombinase activity acting at cer: however this would appear not to be the case. The possibility of cer encoding some regulatory element, such as an RNA or a polypeptide, has not been eliminated.

In conclusion, ColE1 utilizes a site-specific recombination system to monomerize any multimers formed. This system has been postulated to maximise the number of independently segregating plasmid copies within dividing cells and hence increase stability (Summers and Sherratt, 1984). The cer site has been localized to a 280bp region of ColE1 and at least two host gene products are required for cer-mediated recombination. These host functions have been called Xer functions, one of which is probably the recombinase. The relationship between multimerization and instability demonstrated for pACYC184 (Summers and Sherratt, 1984) also, from our data, holds for ColE1.

CHAPTER 4

DERIVATION AND CHARACTERIZATION OF *XER* MUTANTS.

4.1 Introduction

In the last chapter it was established that host functions were required for the *cer*-specific recombination system responsible for enhancing the stability of ColE1. To investigate the host functions necessary for recombination at *cer*, chromosomal mutants defective in such were required.

These mutants enabled us to answer some of the questions addressed in Chapter 3, such as: is there a correlation between multimerization and instability for ColE1? Can ColE1 supply in *trans* the functions absent in the *xer*$^-$ hosts and is there a plasmid function acting at *cer*? The *xer*$^-$ mutants were also used to obtain more information about the *xer* encoded functions, such as the phenotype of *xer*$^-$ strains; the number of complementation groups there are and where these functions map on the *E.coli* K-12 genetic map. These mutants would ultimately enable one to clone the *xer* genes, sequence them, and to set up an *in vitro* system for *cer*-specific recombination.

4.2 Choice of mutagen used to derive *xer* mutants.

A number of treatments cause increased mutation frequency, including ultra violet light, chemical agents and biological mutagenesis. A biological mutagenesis procedure, using the transposon Tn5, delivered into the cell on a lambda vector, was used, because:

(i) The transposon Tn5 carries resistance to a number of aminoglycoside antibiotics, including kanamycin, which allows direct selection of mutants by selection of Kan^r clones.

(ii) The transposon Tn5 has no sites for the restriction endonucleases *Eco*RI, *Bal*I, *Kpn*I, *Pvu*I, *Cla*I and *Sst*I, so that once Tn5 has been shown to genetically mark the gene of interest, it is possible to shot-gun clone from the chromosomal DNA of such a mutant, selecting Kan^r clones, which will contain Tn5 and the flanking sequences of the gene of interest. This flanking DNA can be used as a probe to "walk" along the chromosome, picking up the rest of the gene and neighbouring genes.

(iii) As Tn5 encodes Kan^r, it is possible to move the Tn5 mutant phenotype by P1 transduction to different strain backgrounds, for example one could P1 transduce the mutant phenotype to an appropriate Hfr strain by selecting for transductants which were Kan^r and then use this strain to map the position of this mutation on the genetic map of *E.coli* K-12.

(iv) Tn5 transposes into the chromosome in a non-specific manner so that any gene has a chance of being mutagenized (Berg, 1977), and indeed there are multiple insertion points within a gene.

The genotype of the mutagen used was λb221::Tn5 (Berg, 1977). The b221 deletion removes the phage attachment site, rendering the phage genome incapable of integrating into the host chromosome by lambda-specific mechanisims, however the phage can still grow lytically. Greater than 98% of the Kan^r transductants

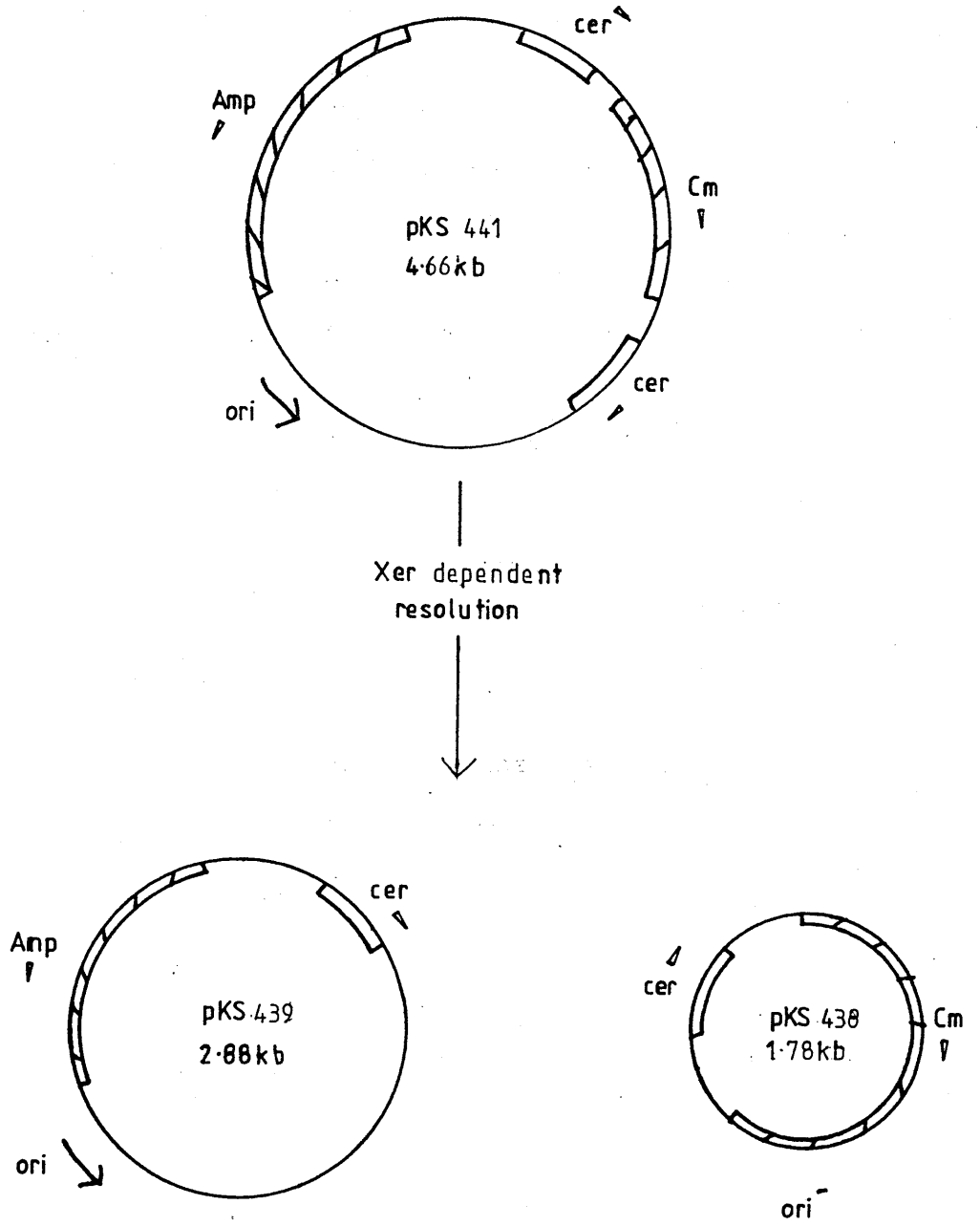


Figure 4.1 Organization of the pseudo-cer dimer, pKS441.

do not contain a prophage and therefore must be formed by transposition of Tn5 from its vector to the E.coli chromosome (Berg, 1977). Berg also reported that the 1-2% of transductants that contain the prophage are unstable and spontaneously lose lambda at a frequency of 10^{-2} to 10^{-3}. The frequency of transposition of Tn5 from one chromosomal site to another is similar to the frequency of transposition during infection, which is approximately 10^{-2} to 10^{-3}. The frequency of reversion of any mutation resulting from Tn5 excision is 10^{-4} to 10^{-6} and nearly all the revertants are Kan^s. The excision of Tn5 is detectable as reversion and is not associated with transposition of Tn5 to a new site.

4.3 Selection procedure for *xer* mutants.

The next problem to be considered in deriving *xer*$^-$ mutants was their detection. The approach used was to transform pools of mutants with a pseudo-*cer* dimer and select/screen for *xer*$^-$ mutants. The pseudo-*cer* dimer used was pKS441 (Figure 4.1). This plasmid has the chloramphenicol resistance gene cloned between two *cer* sites and a second gene, coding for ampicillin resistance, elsewhere on the plasmid. It was not possible to select for recombinase deficient mutants in one step using ampicillin and chloramphenicol, as the parental recombinase proficient host containing pKS441 is Amp^r and Cm^r for a number of generations, even although on single colony gels only the deletion form of the plasmid is visible. This residual chloramphenicol resistance could be due to the production of origin-less Cm^r circles, as the chloramphenicol gene in this construct has its own promoter. These origin-less Cm^r circles may take a number of generations to segregate out, therefore a screen for the levels of CAT synthesis and segregation of $CAT^{+/-}$ phenotypes was required. To screen large populations of mutants

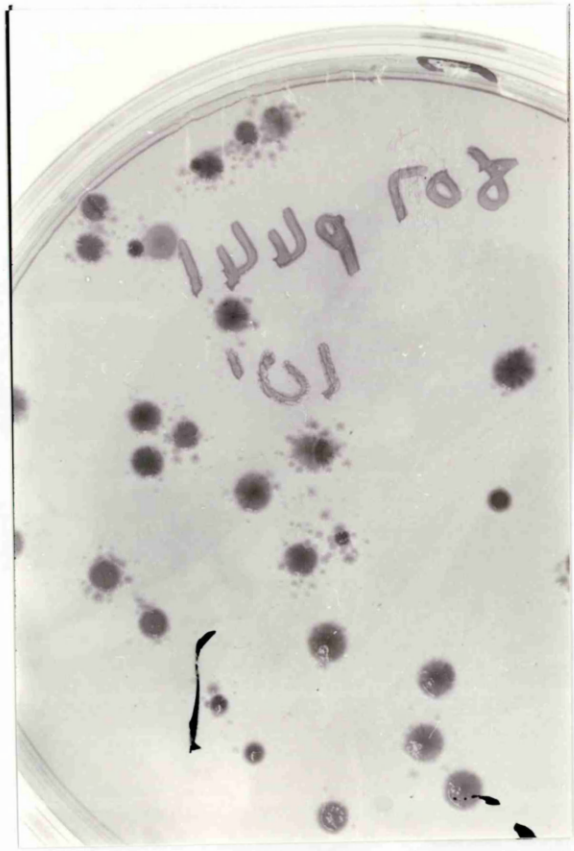


Figure 4.2 Sectoring of colonies caused by segregation of the parental and deletion forms of pKS441 on minimal media + crystal violet.

transformed with pKS441, the rosanilin indicator dye crystal violet was used (Proctor and Rownd, 1982). This dye allows us to detect the levels of CAT synthesis in any colony and segregation of Cm^r in a clone of cells, by a colour plate test. On solid media containing crystal violet, cells which are Cm^r form darker purple colonies than those cells which are Cm^s. This plate test gives a spectrum of shades from purple to white, depending upon the proportion of parental to deletion pKS441 present in a cell. Often sectoring of colonies can be seen, where segregation is occurring (Figure 4.2). These differences in colour are due to the binding of the dye to chloramphenicol acetyl transferase (Tanaka et al 1971, 1974).

Thus mutants affecting cer-specific recombination were to be derived in a two stage procedure: by selecting Cm^r colonies on L-agar + Cm and then screening for levels of CAT synthesis using the crystal violet plate test.

4.3.1 Optimization of the crystal violet plate test.

As the crystal violet plate tests was to be the indicator of xer status in the selection/screening procedure, the test was optimized. It was found that the use of minimal media as opposed to L-agar increased the contrast between CAT^+/CAT^- cells. Some of the parameters which are suggested by Proctor and Rownd (1982), to affect the crystal violet plate test were varied. The first of these parameters was the choice of carbon-source. Proctor and Rownd (1982) stated that fermentable carbohydrate inhibits the colour reaction, whereas non-fermentable carbohydrate does not, this is because CAT synthesis is subject to catabolite repression (Magasanik, 1961). The carbon-source, in the minimal media was varied, comparing a fermentable and non-fermentable source, but no appreciable difference in contrast was observed. This may be due to the fact that in minimal media a

Crystal violet concentration (μg/ml)	Colour contrast	Growth
1·65	+	+ +
1·8	+ + +	+ +
1·95	+ +	+
2·25	+	+
2·4	+	+/−

Table (1)

pH	Colour contrast	Growth
6	+	+
7	+	+ +
8	+/−	+/−

Table (2)

Figure 4.3 Optimum conditions for the CAT, crystal violet plate test.

Table (1) Affect of the crystal violet concentration.

Table (2) Affect of pH.

	Mutant pool A	Mutant pool B
Kan^r colonies (/ml)	1×10^6	$1{\cdot}2 \times 10^5$
Viable count (/ml)	$1{\cdot}5 \times 10^8$	$1{\cdot}4 \times 10^8$
Freq of Tn5 transposition	$0{\cdot}6 \times 10^{-3}$	$0{\cdot}8 \times 10^{-3}$

Figure 4.4 Frequency of Tn5 transposition.

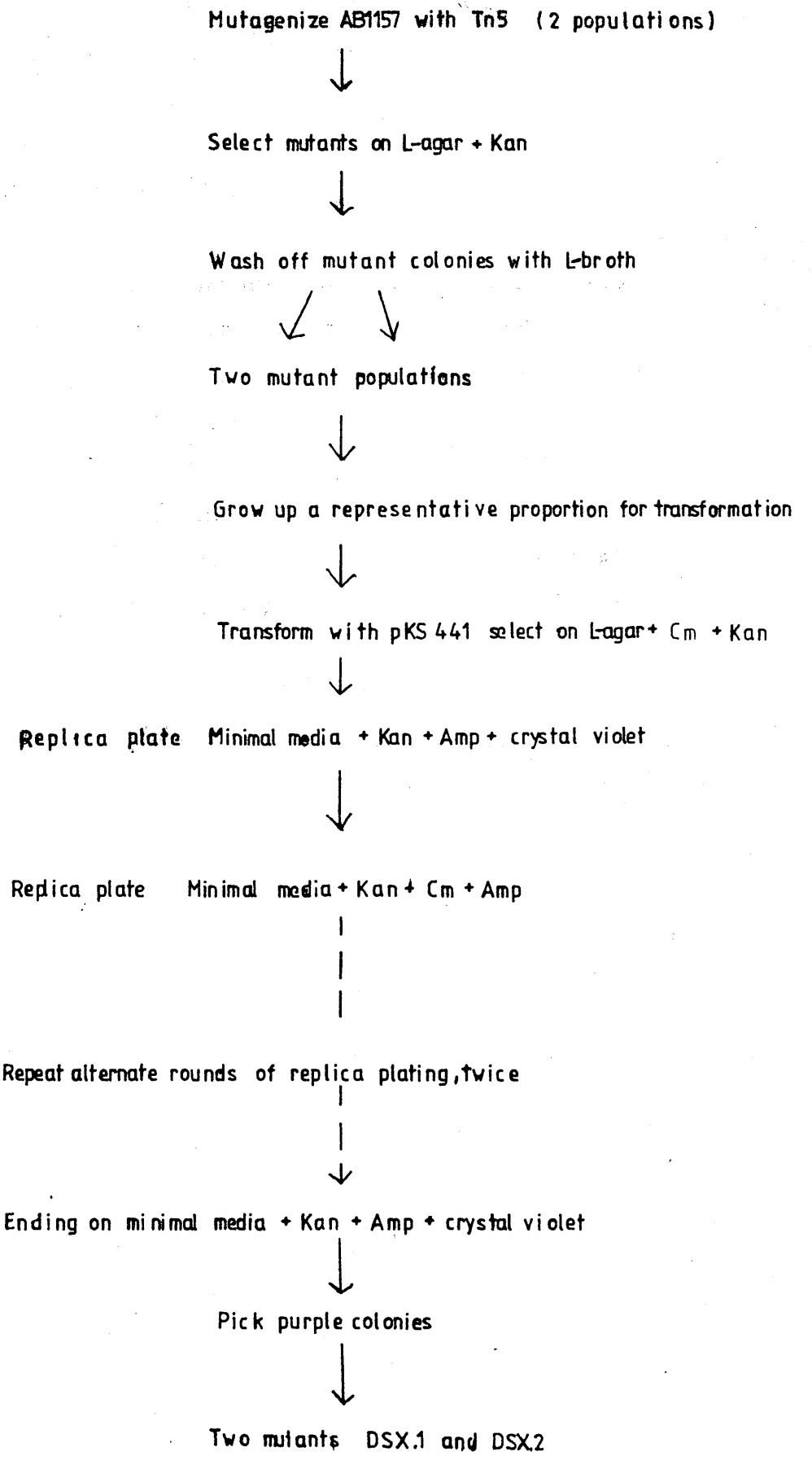


Figure 4.5 Flow diagram illustrating the mutant selection procedure.

very low concentration of C-source i.e. 2g/litre is used, whereas in complex media, such as L-agar a concentration of 10g/litre of glucose is standard. Proctor and Rownd (1982), suggest at least 10g/litre of fermentable C-source is required before CAT^+ colonies fail to absorb the rosanilin indicator dye. The second parameter varied was the concentration of crystal violet in the plates. In L-agar the optimum concentration of crystal violet was 2.25ug/ml; however, in minimal media the contrast was improved, using a lower concentration. At higher concentrations the growth of the cells tended to be reduced as crystal violet is a growth inhibitor (Tanaka, 1971). Therefore a a final concentration of crystal violet of 1.8ug/ml was used. The third variable tested was pH (Figure 4.3). It was concluded that the optimal conditions for our minimal media, crystal violet plate test were:

(i) glucose at a low level 2g/litre

(ii) crystal violet at 1.8ug/ml

(iii) growth media at a pH of 7.0

4.3.2 Derivation of *xer* mutants.

Two populations of the starting strain, DS.903 were mutagenised with λ::Tn5. The two populations were then plated on L-agar + Kan at 20ug/ml to select for mutants and a proportion were plated on L-agar to calculate a viable count, from these figures the frequency of Tn5 transposition was calculated (Figure 4.4). The mutant population was washed off the plates with L-broth. A proportion of this mutant population was then transformed with the test plasmid, pKS441 and plated onto L-agar plates + Kan + Cm. The transformants then went through a series of alternative replica platings rounds on minimal media + Cm + Kan + Amp and then minimal media + Amp + Kan + crystal violet, finishing on minimal + Amp + Kan + crystal violet (Figure 4.5). Throughout

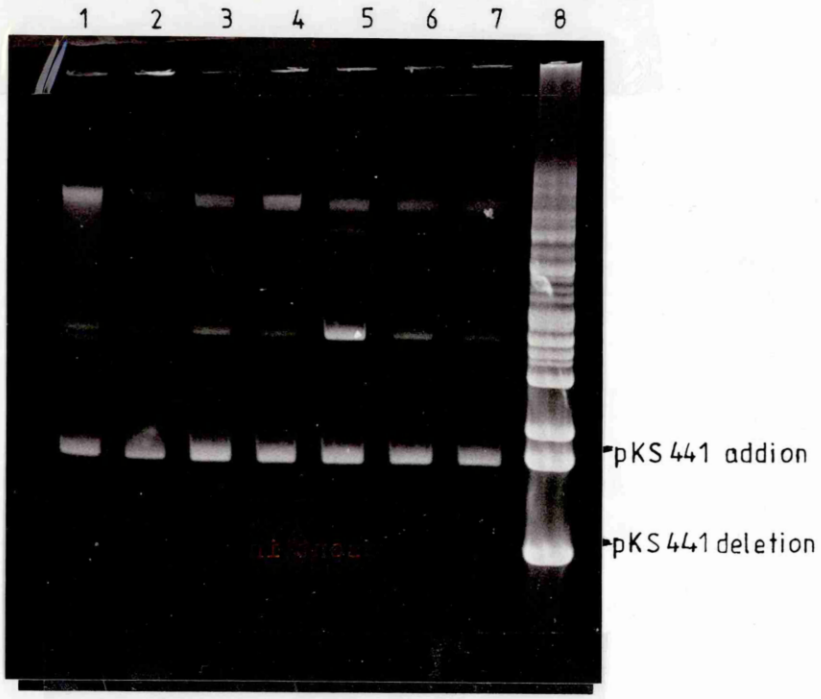


Figure 4.6 A single colony gel of the mutants DSX.1 and DSX.2 containing pKS441.

Lanes (1-3) pKS441 in DSX.1

(4-7) pKS441 in DSX.2

(6) mixed DNA preparation containing pKS441 and pKS442

these rounds of replica plating Amp selection was maintained to retain pKS441 in the mutant population. The alternate plating rounds on chloramphenicol, selected for mutants unable to break down pKS441. At the last replicate any dark purple colonies, which had arisen were selected and streaked out to single colonies on minimal media + Amp + Kan + crystal violet. At this stage these colonies were examined carefully for segregation of the purple colonies to white as this would imply segregation of a Cm^r and Cm^s phenotype. A binocular microscope, was used to look for any segregation, but none was observed. At this point there were two possible mutants: single colonies of each were picked and patched on minimal media + Amp + Kan + crystal violet. These patched single colonies were then examined on single colony gels to check the status of pKS441 (Figure 4.6). Examination of these gels confirmed that there was no segregation occurring and that the pKS441 was still present in the parental form in these two mutants. At the end of that selection procedure two mutants were independently derived, each from a different mutant population, allowing one to say that these two mutants are not siblings. These two mutants were called DSX.1 and DSX.2.

4.4 Are the mutants DSX.1 and DSX.2 plasmid or chromosomal mutants?

To determine if these two mutants were plasmid rearrangments in pKS441 or host mutants affecting cer-specific recombination, the status of pKS441 had to be determined. If the pKS441 in DSX.1 and DSX.2 was normal then it would be broken down to the deletion form by the parental xer$^+$ strain, DS.903. DNA of pKS441 from DSX.1 and 2 was made by the alkaline SDS denaturation procedure (Birnboim and Doly, 1979) and transformed into the parental xer$^+$ host, DS.903. Transformants were selected on L-agar + Amp + crystal violet, and subsequently picked and patched for

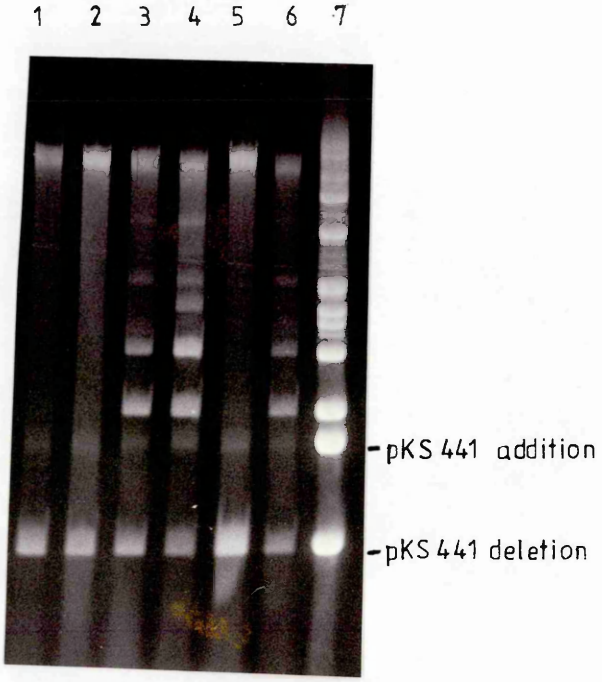


Figure 4.7 Plasmid DNA of pKS441 isolated from DSX.1 and DSX.2, re-transformed into DS.903.
Note that the parental pKS441 isolated from DSX.1 and 2 is broken down to the deletion form in DS.903.

Lanes (1-3) pKS441 from DSX.1 in DS.903
(4-6) pKS441 from DSX.2 in DS.903
(7) mixed DNA preparation containing pKS441 and pKS442

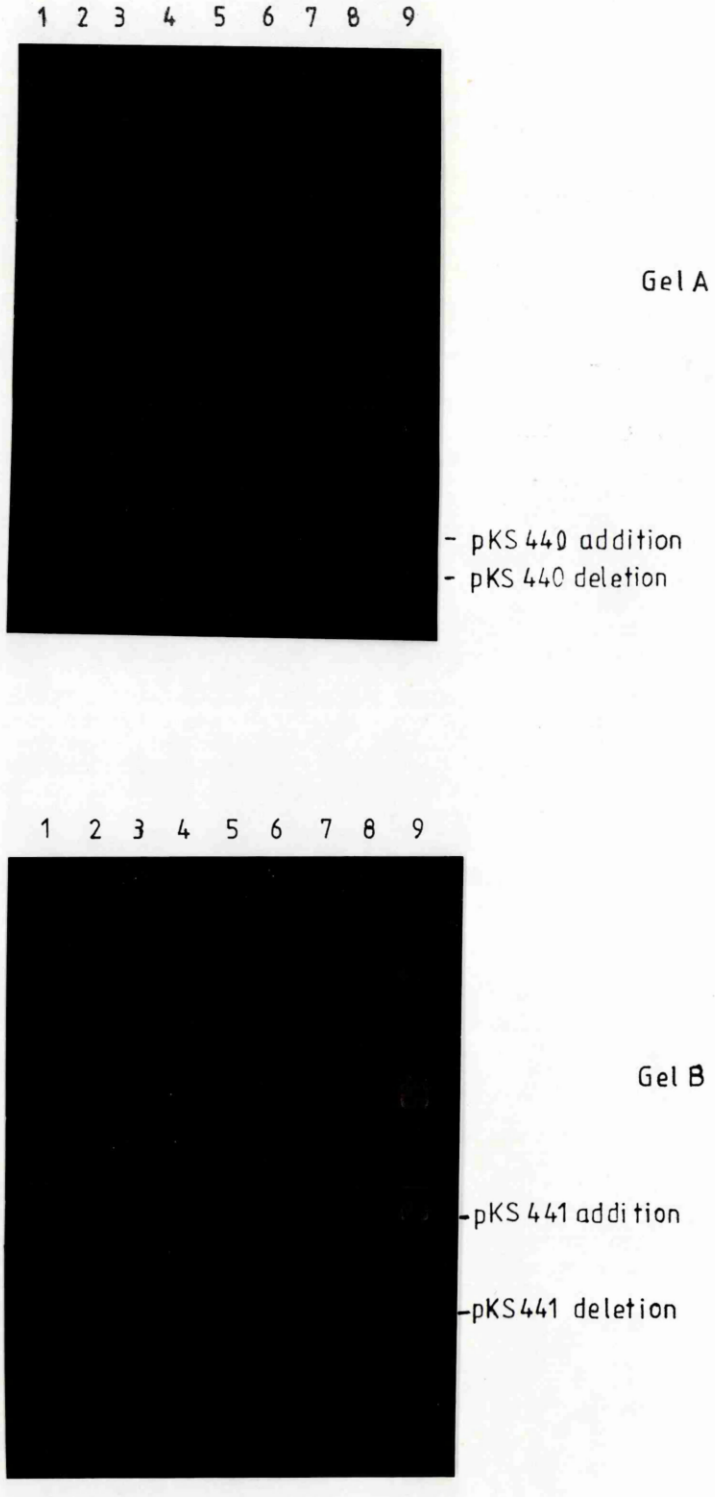


Figure 4.8 Plasmid-free derivatives of DSX.1 and DSX.2 transformed with the pseudo-ger dimers, pKS440 and pKS441.

Gel (A)

Lanes (1-4) pKS440 in DSX.1

(5-7) pKS440 in DSX.2

(8) pKS440 deletion derivative

(9) pKS440 addition

Gel (B)

Lanes (1-4) pKS441 in DSX.1

(5-8) pKS441 in DSX.2

(9) mixed preparation of pKS441 and pKS442

examination on single colony gels (Figure 4.7). These gels show that the pKS441 DNA made from DSX.1 and 2 was broken down to the deletion form by the parental strain, DS.903. This observation implies that DSX.1 and 2 are not plasmid rearrangements of pKS441 but are host mutations affecting cer-specific recombination.

To confirm that DSX.1 and 2 are host mutants affecting cer-specific recombination, plasmid free derivatives were derived and re-transformed with pseudo-cer dimers. Plasmids which are cer$^-$ (such as pUC8) are relatively unstable in xer$^+$ strains, therefore one would postulate that cer$^+$ plasmids in a xer$^-$ host would also be unstable. Based on this hypothesis plasmid-free derivatives were derived by growing these two strains, DSX.1 and 2, in minimal liquid media without glucose, overnight. These cells were then plated out onto L-agar + Kan and subsequently screened on L-agar + Kan and L-agar + Kan + Amp, for cells which had lost pKS441. The plasmid-free cells will grow on L-agar + Kan but not on L-agar + Kan + Amp and approximately one half of the cells tested were plasmid-free. This result was supported by looking at the plasmid-free isolates on single colony gels.

The plasmid free derivatives DSX.1 and 2 were retransformed with two cer test plasmids, pKS441 and pKS440. These transformants were selected on L-agar + Amp, and examined on single colony gels (Figure 4.8). These gels show pKS440 and pKS441 in their parental form in DSX.1 and 2, confirming that these two mutants lack host functions affecting cer-specific recombination.

4.5 Co-transduction of Kan^r and the Xer$^-$ phenotype.

Markers which are closely linked on the E.coli chromosome i.e.within 1.8 minutes, can be co-transduced in the same P1 phage particle (Miller, 1972). If a P1 lysate is made on a xer$^-$ strain and used to P1 transduce into a xer$^+$, Kan^s strain, selecting for Kan^r clones one should isolate transductants which are xer$^-$, at a

high frequency if Tn5 is in or close to a xer gene. It was discovered in attempting these experiments that DSX.1 does not plate P1. DSX.2, however does plate P1, so a P1 lysate was made from this strain and used to P1 transduce DS.903 to Kan^r. Twenty such Kan^r transductants were transformed with pKS441 and all twenty were found to break pKS441 down to its deletion form i.e. all twenty were xer^+. The inability to co-transduce Kan^r and a Xer^- phenotype, imply that Tn5 is not in or near the mutant xer gene in DSX.2. The mutation in DSX.2 affecting cer-specific recombination is therefore probably a spontaneous mutation.

4.5.1 Ability of DSX.1 and 2 to plate lambda.

The inability of DSX.1 to plaque P1 prompted us to look at the ability of DSX.1 and 2 to plaque lambda. To test if DSX.1 and DSX.2 are lambda lysogens or are $lambda^r$ two lambda phages were used λvir and λcI. λvir will grow on E.coli K-12 regardless of whether or not it is a lysogen, whereas λcI will not grow on a lysogenic host. Both DSX.1 and 2 fail to plaque with λvir and λc1, which implies that lambda is unable to get into these strains at all, perhaps due to a cell wall mutation. DSX.1 is also Mal^- in contrast to the Mal^+ parental strain DS.903. Most mutations in E.coli K-12 to λ^r map at two loci: malA at 75 minutes and malB at 91 minutes (Randall-Hazelbauer and Schwartz, 1973). It would appear that DSX.1 is λ^r, Mal^- and that the mal mutation maps to malB, whereas DSX.2 is λ^r and Mal^+.

Why have λ^r mutants been generated? Our procedure of λ::Tn5 mutagenesis itself generated 5% Mal^-,λ^r clones. If one is not meticulous with the washing procedures this can increase to 25% Mal^-, λ^r. Thus the isolation of λ^r mutants is probably caused by the mutagenesis procedure itself. This is one of the problems of not using a defective lambda phage. To get around the problem of creating λ^r strains, a defective lambda phage was used by

Colin Stirling to derive subsequent xer::Tn5 mutants. The system used was λ467 (Kleckner, 1977), the genotype of which is b221, rex::Tn5, cI857, Oam29, Pam80. The b221 mutation removes the attachment site, preventing the bacteriophage from undergoing lysogeny. The rex gene, is non-essential for growth and contains the Tn5 insertion. The cI587 mutation gives this phage a temperature sensitive cI repressor. Both the O and P genes are involved in replication so amber mutations in a Sup^{o} background will prevent phage replication. Using λ467 ten xer mutants have been derived, one of which, DSX.300 appeared to be a host mutation affecting cer-specific recombination. In P1 transduction experiments, using a lysate made on DSX.300, twenty Kan^{r} transductants were also Xer^{-} in phenotype. Showing that there is high linkage between Kan^{r} and Xer^{-} phenotype, impling that Tn5 is inserted in or near a xer gene in the mutant strain, DSX.300.

4.6 Maintenance and stability of multimers of cer^{+}, ckr^{+} and crl^{+} plasmids in $xer^{+/-}$ hosts.

In sections, 4.6.1 and 4.6.2, the ability of xer^{+} and xer^{-} strains to monomerize $cer^{+/-}$ plasmids and the stability of these plasmids were examined. At the same time plasmids containing sites analogous to cer, including pRY10 containing the ckr site of ColK, and pEV87 which contains the crl site of CloDF13, were examined for stability and multimers in xer^{-} and xer^{+} hosts, in order to determine if ColK and CloDF13 use xer functions.

```
          3731
COLE1     GTGAAACCATGAAAAATGGCAGCTTCAGTGGATTAAGTGGGGGTAATGTGGCCTGTACCC
PMB1      .C.....T.............G...G.......TT.AC...C........T...C...A
COLK      TGA...GATG......AAA...TG...TGA.G..T.CGCA..CA..ACCA..G.TATT.A
CLODF13   AGA..GT.GGT.....AA.GGCT.A.G.CATCCATTT.AC.TCA..ACATATGCTATG.A


COLE1     TCTGGTTGCATAGGTATTCATACGGTTAAAATTTATCAGGCGCGATCGC-G-CAGTTTTT
PMB1      ..............................................G..........
COLK      CA...C..A....C...G.....................T..........G...-....
CLODF13   GT..CG........C..G...TA..A.........C.G......T.TC....GC.G....


COLE1     C-GGGTGGTTTGTTGCCATTTTTACCTGTCTGCTGCCGTGATCGCGCTGAACGCGTT-TT
PMB1      ..............................................A.............
COLK      ..............................A..A.....................A....C
CLODF13   .C......G.......TTG.....T.CCGTA..C....GA.A...CGCC.GT..C..AC.

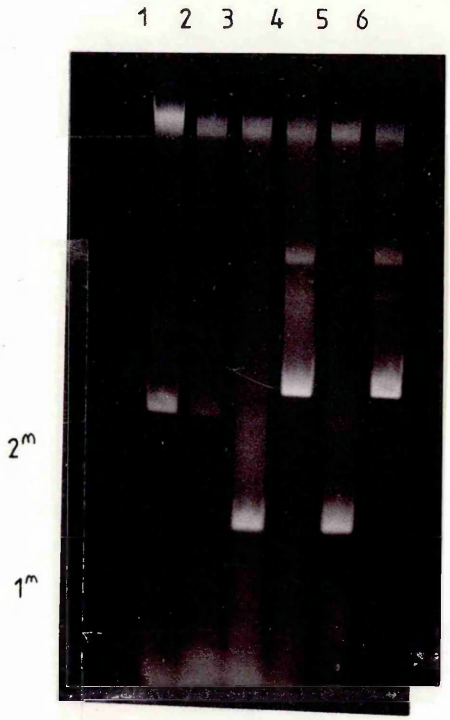

COLE1     AGCGGTGCGTACAATTAAGGGA--TTATGGTAAATCCACTTACTGTCTGCCCTCGTAGCC
PMB1      ....................................A.ACGTA...TAAT.TATCG.CAT
COLK      .....................................--..................T..
CLODF13   G.....-----..CGA.......TG...........ATCT.CGAGT.TCAGGTA.AA.AAA

             3969
COLE1     ATCGAGA
PMB1      ..GT.AC
COLK      .......
CLODF13   .G.TCCG
```

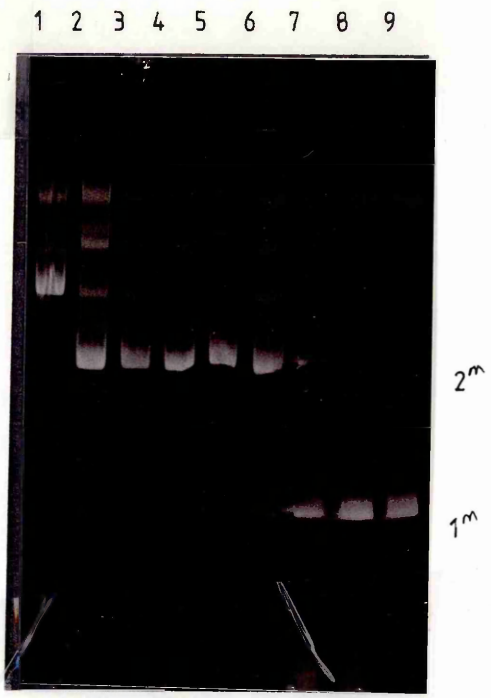
Figure 4.9 Sequence comparison of the sites analogous to ger from pMB1, ColK and CloDF13.

(.) represents homology

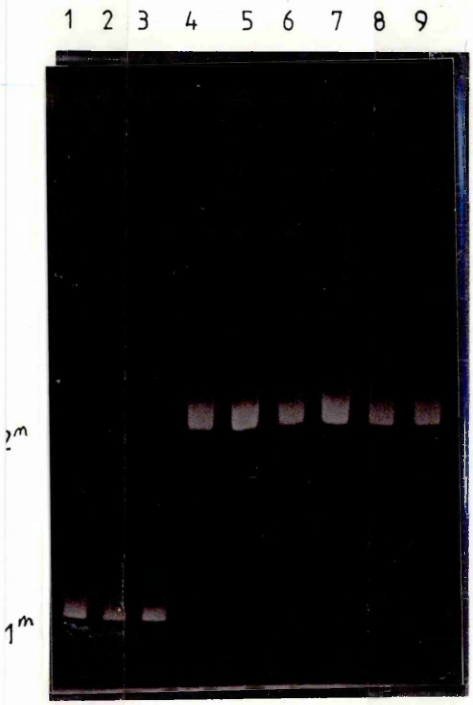
(-) represents a gap



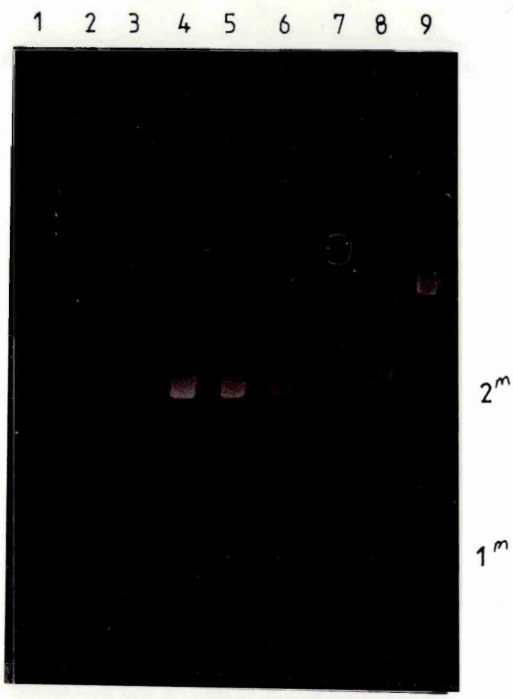
Gel A



Gel B



Gel C



Gel D

Figure 4.10 Isogenic *xer*$^+$ and *xer*$^-$ strains transformed with multimeric forms of pUC8, pKS450, pRY10 and pEV87.

Gel (A)

Lane (1) pUC8 dimers in DS.903

(2) pUC8 dimers in DSX.1

(3) pKS450 dimers in DS.903

(4) pKS450 dimers in DSX.1

(5) pRY10 dimers in DS.903

(6) pRY10 dimers in DSX.1

Gel (B)

Lanes (1-3) pEV87 dimers in DSX.1

(4-6) pEV87 dimers in DSX.2

(7-9) pEV87 dimers in DS.903

Gel (C)

Lanes (1-3) pKS450 dimers in DS.903

(4-6) pKS450 dimers in DSX.1

(7-9) pKS450 dimers in DSX.2

Gel (D)

Lanes (1-3) pRY10 dimers in DS.903

(4-6) pRY10 dimers in DSX.1

(7-9) pRY10 dimers in DSX.2

4.6.1 The ability of *xer*$^-$ strains to maintain multimers of *cer*$^+$, *ckr*$^+$ and *crl*$^+$ plasmids.

The natural plasmid ColE1 is normally stable, as it contains a site *cer*, at which a recombinase, probably of host origin, acts converting multimers to monomers, hence increasing stability. If a *xer*$^-$ host is transformed with multimers of plasmids containing a *cer* site, these should remain multimeric and be unstable.

The analogous sites in ColK and CloDF13, called *ckr* and *crl* are 90% and 69% homologous respectively to a 150bp core region of *cer* (Figure 4.9). These sites, may be involved in an analogous stability system to that used by ColE1. Therefore, the host *xer* functions, which appear to be used by the *cer*-site specific recombination system may also be used by ColK and CloDF13. If this is so, then in *xer*$^-$ hosts plasmids containing the site analogous to *cer*, *ckr* and *crl* will be maintained as multimers and should be unstable. Dimers of various plasmids were gel purified from the hyper-recombinogenic strain, JC8679. These plasmids were pUC8 (containing no *cer* site), pKS450 (containing *cer*), pRY10 (containing *ckr* of ColK) and pEV87 (containing *crl* of CloDF13). The purified dimeric DNA was transformed into DS.903, DSX.1 and DSX.2 and examined on single colony gels (Figure 4.10). From these gels it was observed that pUC8 stays dimeric in *xer*$^+$ and *xer*$^-$ strains, as it contains no *cer* site or equivalent; whereas pKS450 containing a *cer* site is broken down in a *xer*$^+$ strain, DS.903 but not in DSX.1, the *xer*$^-$ strain. Similar results were obtained for pRY10 containing *ckr* and pEV87 containing *crl*. That is to say that pRY10 and pEV87 were maintained as multimers in a *xer*$^-$ host but were monomerized in a *xer*$^+$ host.

These results imply that the multimer resolution systems of these three natural plasmids ColE1, ColK and CloDF13 all utilize the same *xer* host functions. The homology between the *cer* site of ColE1 and the *ckr* site of ColK is 90% over a 150bp core region

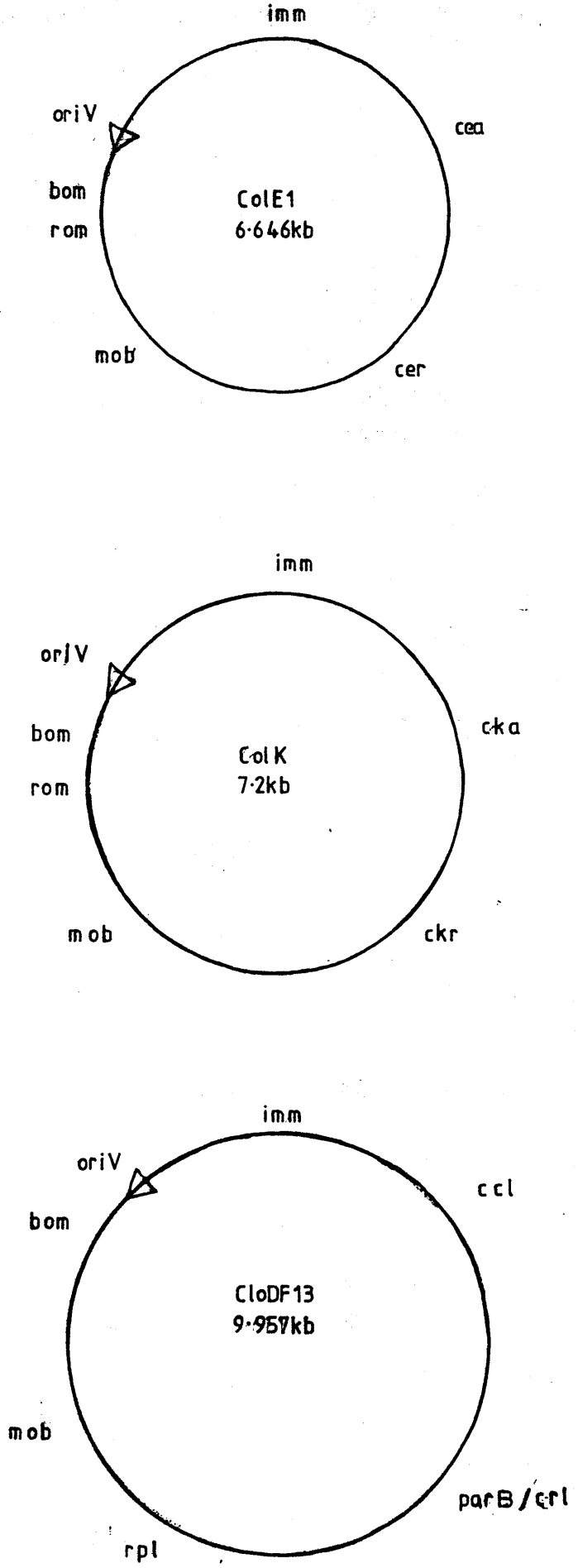


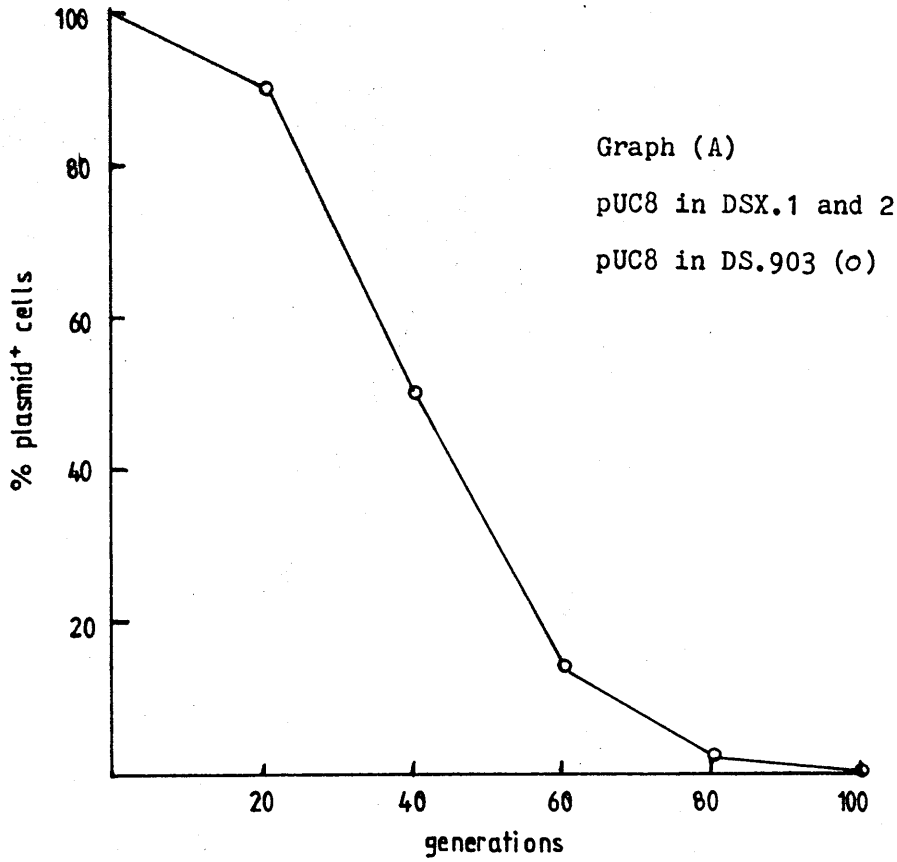
Figure 4.11 Structural comparison of ColE1, ColK and CloDF13.

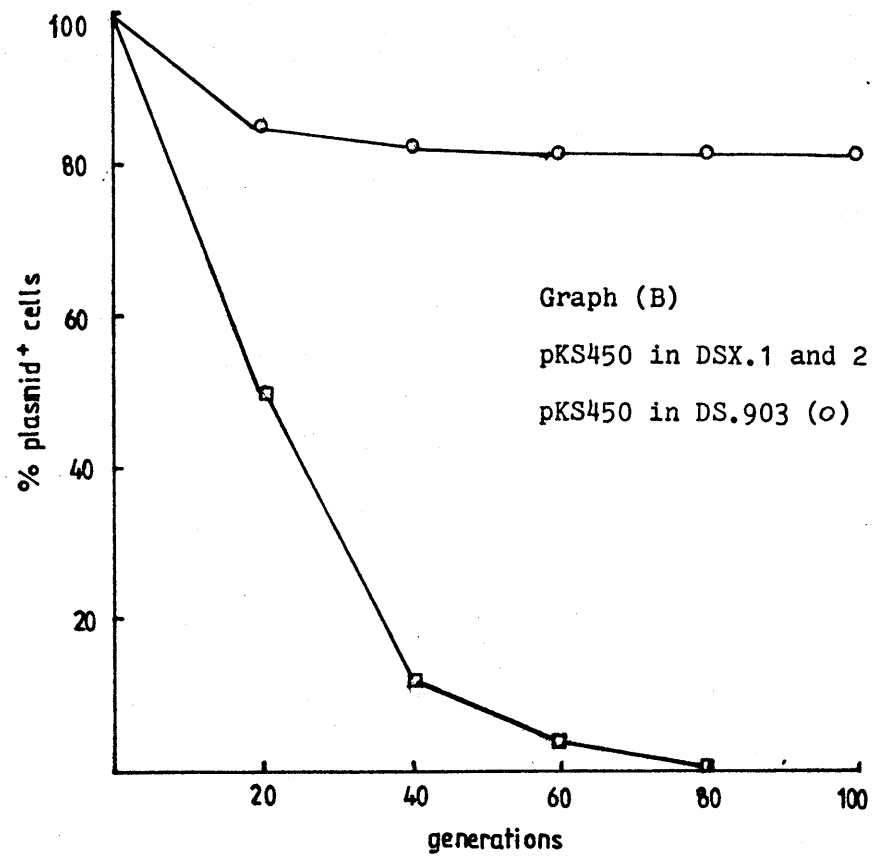
(Summers et al, 1985), whereas crl of CloDF13 has only 69% homology to the core region of cer (Sherratt et al, 1984). The divergence between these sites is consistent with the relationship suggested by structural and functional comparisons of the plasmids (Figure 4.11). ColK is compatible with ColE1 but encodes mobility functions that complement those of ColE1. colicin E1 and colicin K are also similar in that they both act upon the cytoplasmic membrane (Broda, 1979). Whilst the overall organization of ColE1 and CloDF13 is similar (Veltkampe and Stuije, 1981), the plasmids are compatible and cloacin DF13 does not resemble colicin E1 in its mode of action. CloDF13 is also maintained at a lower copy number of 10 per cell (Veltkampe et al, 1974), compared to 20 copies per cell for ColE1 (Timmis, 1981). The natural plasmids ColE2 and ColE3 have also been shown to form multimers in xer$^-$ hosts (M. Burke pers. comm), implying that ColE2 and ColE3 may also utilize xer functions to maintain their stability.

4.6.2 The stability of cer$^+$, ckr$^+$ and crl$^+$ plasmids in xer$^+$ and xer$^-$ strains.

In the experiments just discussed, xer$^-$ strains maintain multimers of cer$^+$, ckr$^+$ and crl$^+$ plasmids whereas, xer$^+$ strains break these multimers down. One would predict that the inability to monomerize plasmid multimers in xer$^-$ hosts would result in a decrease in plasmid stability. The stability of these plasmids was examined in xer$^+$ and xer$^-$ strains, using the B-lactamase plate test of Boyko and Ganschow (1982). This test could not be used to assess the stability of the ColE1 Amp^r plasmids in xer$^-$ hosts as the copy number was low. The plasmids used in this experiment have a higher copy number, therefore it was valid to use the B-lactamase test in this instance. The stability of pUC8, pKS450 (containing cer), pRY10 (containing ckr) and pEV87

Figure 4.12 Stability of pUC8, pKS450, pRY10 and pEV87 in DSX.1, DSX.2 and DS.903, under non-selective conditions.

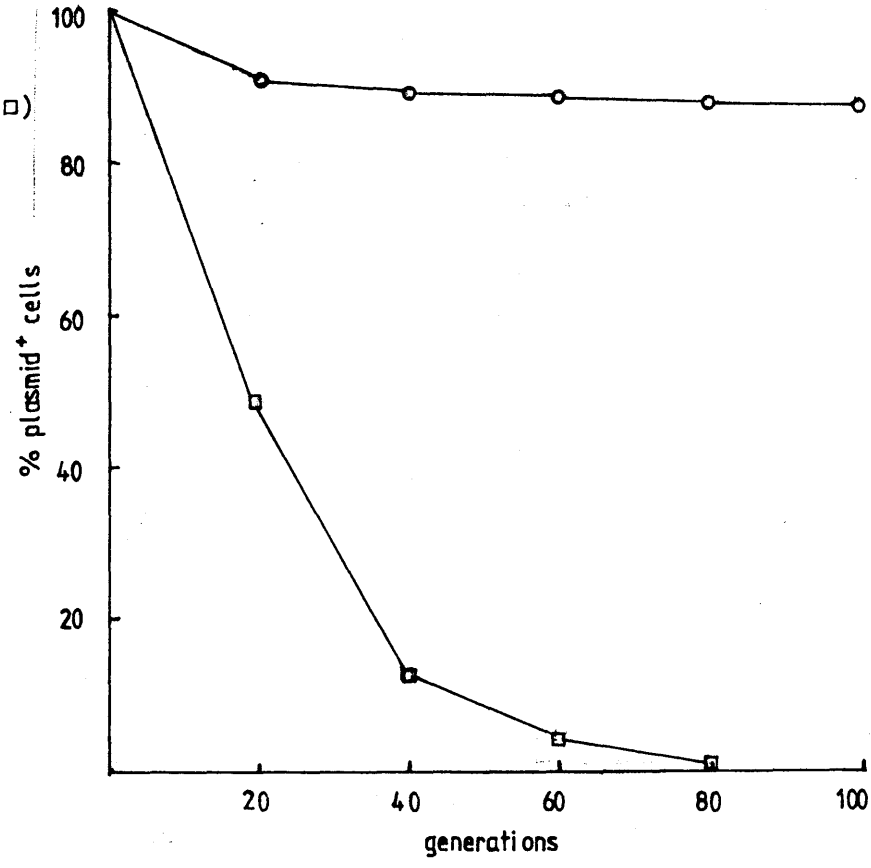




Graph (C)

pRY10 in DSX.1 and 2 (□)

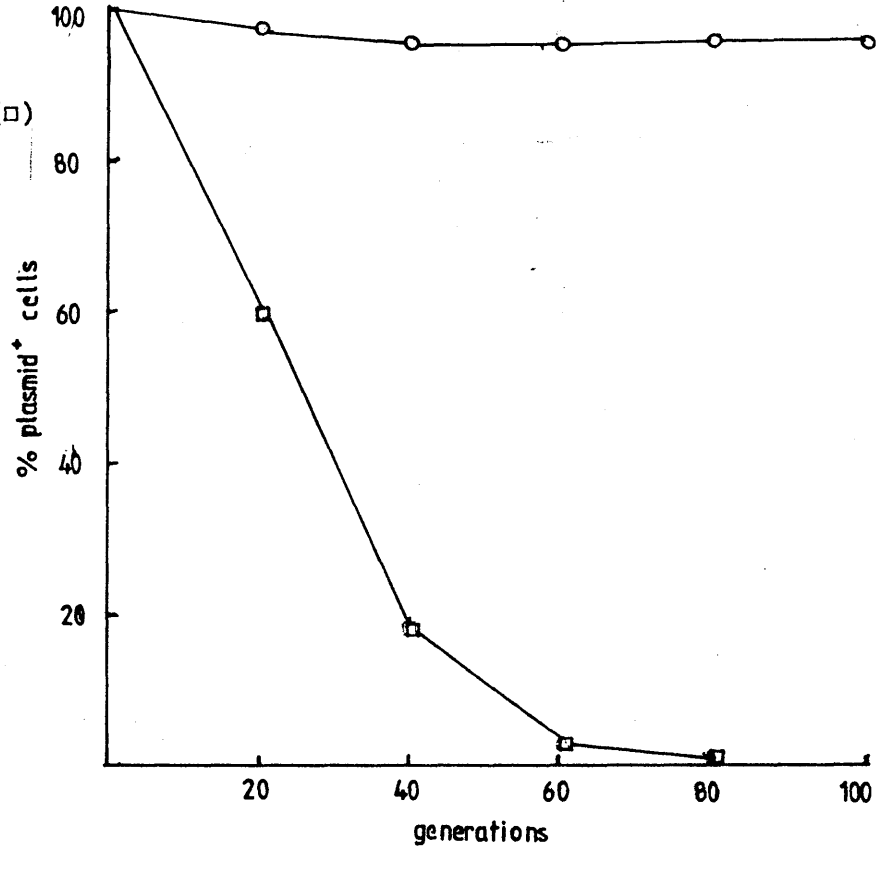
pRY10 in DS.903 (o)



Graph (D)

pEV87 in DSX.1 and 2 (□)

pEV87 in DS.903 (o)



(containing *crl*); were examined in isogenic *xer*$^+$ and *xer*$^-$ strains at 0 to 100 generations, sampling every 20 generations. At each time point the percentage plasmid$^+$ cells was calculated using the plate test for B-lactamase previously mentioned. These data were plotted as percentage plasmid$^+$ cells versus number of generations, for each of the plasmids used (Figure 4.12). From the graphical representations of these data a number of conclusions were reached. Firstly, when a strain is *xer*$^+$ and contains a plasmid which is *cer*$^+$ or has the analogous site from ColK or CloDF13, then these plasmids are stable i.e. pKS450, pRY10 and pEV87 in a *xer*$^+$ host. Secondly, when the host is *xer*$^-$ or when the plasmid has no *cer* site or equivalent, then the plasmids are unstable, as is seen with pUC8 in a *xer*$^+$ or *xer*$^-$ host and pKS450, pRY10 and pEV87 in a *xer*$^-$ host.

In conclusion, in *xer*$^-$ strains, plasmids containing *cer*, *ckr* or *crl* are maintained as multimers and are unstable, whereas in *xer*$^+$ strains these plasmid multimers are broken down to monomers and are stable. The plasmid pUC8, containing no *cer* site is maintained in the multimeric state in *xer*$^+$ and *xer*$^-$ hosts and is unstable in both backgrounds. These results imply that the *xer* functions are required for *cer*-specific recombination to break down any multimers present to monomers. Summers and Sherratt (1984), suggested that any factors causing an increase in multimerization create a decrease in stability. These data support that idea as in *xer*$^-$ strains multimers of *cer*$^+$ plasmids are maintained and are unstable. This also appears to be true of the analogous systems in ColK and ColDF13, which use the sites *ckr* and *crl*. Multimers of *ckr*$^+$ and *crl*$^+$ plasmids are maintained in *xer*$^-$ strains and are unstable, whereas in *xer*$^+$ strains these plasmids are monomerized and stable. These data imply that a site-specific recombination system similar to the *cer*-specific recombination system is involved in the stability of ColK and CloDF13, acting at the *ckr* and *crl* sites respectively.

Figure 4.13 Doubling times of isogenic *xer*$^+$ and *xer*$^-$ strains.

Strain	Doubling time (mins)
DS.903	29
DSX.1	29
DSX.2	30
DSX.330	30

4.7 Analysis of the growth rate of isogenic *xer*$^+$ and *xer*$^-$ strains.

The growth rates of four strains, isogenic apart from their *xer* status were examined to determine if the Xer phenotype has any affect. These strains were DS.903, DSX.1, DSX.2 and DSX.330. A growth curve was plotted for each of these strains and from this, doubling times were calculated (Figure 4.13). There was no significant difference in the growth rates of these four strains. If there was an effect of the *xer* status of the cell on the growth rate one may have to look at less mutant *E.coli* strains to see any difference, such as CSH26 and DSX.360, or alternatively examine the growth rates of a mixed culture of *xer*$^-$ and *xer*$^+$ cells.

Discussion and conclusions.

In Chapter 3 it was established that there was a host component to the *cer*-specific recombination system of ColE1. It is presumed that the host encodes the recombinase acting at *cer*. To investigate this system further *E.coli* K-12 mutants that are deficient for *cer*-specific recombination were sought. These *xer*$^-$ mutants were used to show that there is a correlation between multimerization and instability for ColE1; that ColE1 in *trans* can not supply any of the functions absent in the *xer* mutants and that there are no plasmid functions, acting at *cer*, [although there may be control elements encoded by ColE1 (Chapter 3)]. In this chapter the phenotype of *xer* mutants has been examined. To obtain *xer* mutants Tn5 mutagenisis was used (Berg, 1977). Tn5 was used as it has been shown to exhibit no site specificity in its transposition and has the advantage of carrying the gene for kanamycin resistance, enabling one to genetically mark the gene of interest, which provides a means of cloning that gene. To

select xer mutants our approach was to make populations of Tn5 insertion mutants, then to transform them with pKS441, a pseudo-cer dimer, which has the gene encoding for Cm^r between the two cer sites. Initially choramphenicol was used to select for mutants and then crystal violet (Proctor and Rownd, 1982) was used as a screen, to detect the status of pKS441 (addition/deletion). Two independent mutants were derived thus: DSX.1 and DSX.2. P1 transduction analysis of DSX.2 showed that it is a spontaneous xer mutant. DSX.1 does not plate P1 therefore it is not known if Tn5 is close to the mutant xer gene or if it is a spontaneous mutant. A third mutant which has a Tn5 insertion in a xer gene was derived by Colin Stirling, and is called DSX.300.

DSX.1, DSX.2 and DS.903 were used to investigate the ability of these strains to monomerize cer^+ and cer^- plasmid multimers and the stability of these plasmids in these strains. Plasmid multimers, containing no cer site, such as pUC8, are not monomerized and are unstable in xer^+ and xer^- strains. However, multimers of pKS450, containing a cer site, are monomerized and stable in a xer^+ host; but remain multimeric and are unstable in a xer^- host. These results imply that Xer functions are required for cer-specific recombination, to break down multimers to monomers. In xer^- hosts, cer^+ plasmid multimers are maintained, which one could postulate would result in a decrease in the number of segregating units and hence a decrease in stability, as observed.

ColK and ClODF13 have been shown to have sites, which are analogous to cer, called ckr and crl respectively. The ckr site is 90% homologous cer, whereas crl is 69% homologous to cer. The divergence between these sites is consistent with the relationships suggested by structural and functional comparisons of these plasmids. Hence xer^+ and xer^- strains were examined for their ability to maintain multimers of ckr^+ and crl^+ plasmids

and the stability of these plasmids. Multimers of plasmids containing *ckr* and *crl* were monomerized and stable in *xer*$^+$ hosts but not in *xer*$^-$ hosts. Plasmids containing the *ckr* site of ColK or the *crl* site of CloDF13 behave in a similar fashion in *xer*$^+$ and *xer*$^-$ strains as plasmids containing the *cer* site of ColE1, with respect to multimer maintenance and stability. From these data one can conclude that ColK and CloDF13 use Xer functions to maintain stability in a similar fashion to ColE1. These plasmids use a site-specific recombination system to break down any multimers present, which could be hypothesised to maximise the number of independently segregating units, so increasing their stability. The natural plasmids ColE2 and ColE3 have also been shown to form multimers in *xer*$^-$ hosts, implying that these plasmids may also utilize *xer* encoded functions to maintain their stability.

If more time had been available other aspects of the Xer phenotype could have been examined such as U.V. sensitivity, homologous recombination deficiencies and the dependence of other site-specific recombination systems on the *xer* genes. In the lambda *int* site-specific recombination system a host factor IHF is required (Craig and Nash, 1984). Since the requirement of the lambda *int* system for IHF has been discovered IHF has been shown to be involved in many other systems, such as translation of the *c*II message of lambda, expression of *ilv* and *xyl*, encapsidation of lambda and replication of pSC101 (Gamas *et al*, 1986). Perhaps one or both of the *xer* gene products may also have multiple phenotypes.

CHAPTER 5

CLONING OF THE WILD TYPE *XERA* GENE

5.1 Introduction

The Tn5 insertion mutant, DSX.300 has been shown by P1 transduction of Kan^r to have Tn5 inserted in or very close to a *xer* gene. The Tn5 sequences plus flanking DNA were subsequently cloned out of DSX.300 using *Eco*RI, which does not cut in Tn5. The clone isolated was called pCS300 and contains Tn5 plus 9.3kb of flanking chromosomal DNA. Restriction analysis shows that Tn5 is situated centrally in the flanking chromosomal DNA, implying that the *xer* sequence may be contained in this *Eco*RI fragment. This allowed cloning of the wild type sequence from a xer^+ strain, using *Eco*RI. The wild type clone was called pGS30 and complements the Xer^- phenotype of DSX.360, a $recA^-$ derivative of DSX.300. The wild type clone contains an insert of 8.45kb, which was subsequently reduced in size to a 0.95kb minimum complementing clone, that contains the *xerA* gene.

5.2 Cloning of the mutant *xer* gene from DSX.300.

Colin Stirling was responsible for constructing DSX.300, using λ467 (Kleckner, 1977). In P1 transduction experiments the Kan^r and Xer^- phenotypes were shown in twenty out of twenty cases to be co-transducible markers, implying that Tn5 and the mutant *xer* gene are closely linked. The original mutant strain, DSX.300 and four of the Kan^r P1 transductants were probed in a Southern blot analysis using as a probe pBR322::Tn5. Diagnostic *Bam*HI and *Sph*I bands were found to light up in the original mutant and the four P1 transductants tested. Showing that Tn5 is in the same site and is present in only one copy in the mutant DSX.300 and the four P1 transductants. It was decided to clone the Tn5 sequences from DSX.300, in the hope of selecting some *xer* sequence. Chromosomal DNA of DSX.300 was made and restricted with *Eco*RI, which does not cut in Tn5, generating a population of fragments.

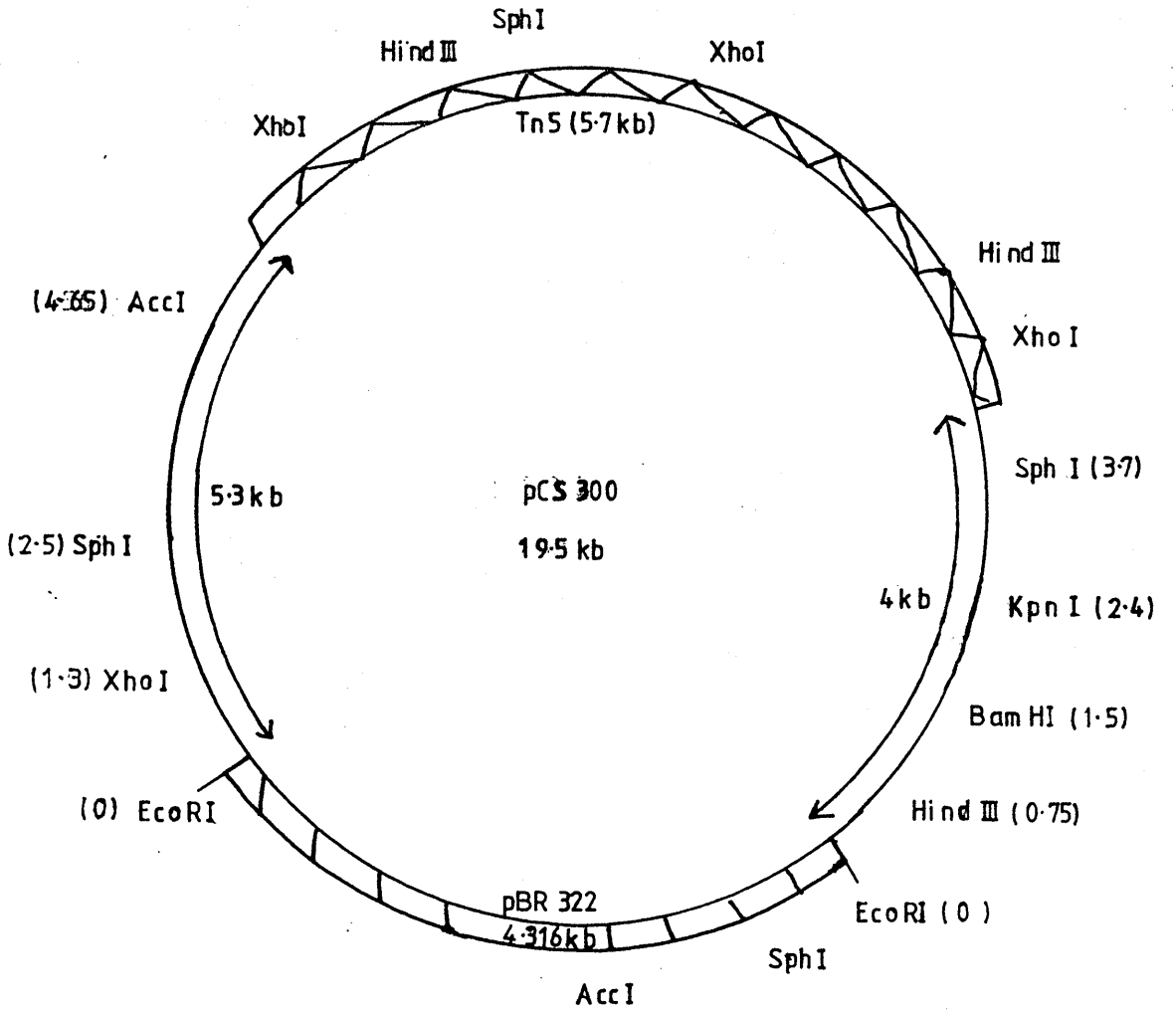


Figure 5.1 The mutant clone, pCS300.

The figures in ( ) represent the distance in kb from the proximal EcoRI ends.

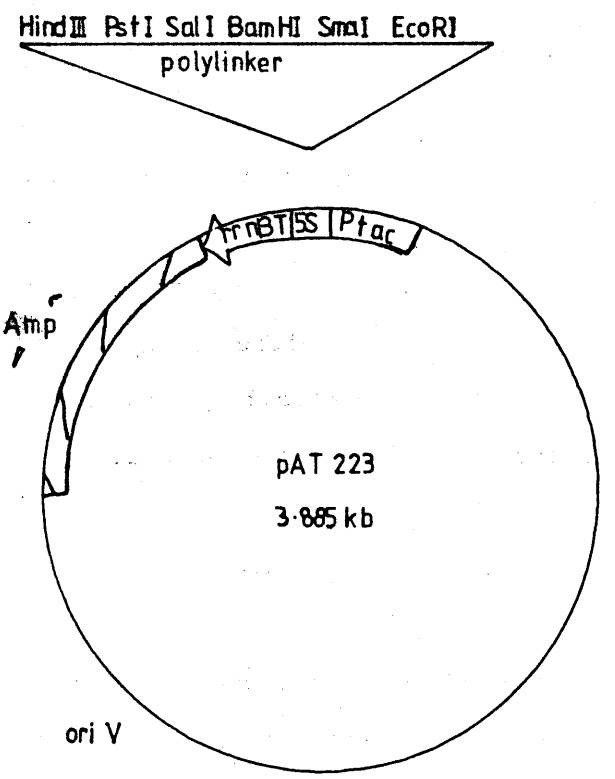


Figure 5.2 The ptac expression vector pAT223.

These fragments were ligated into the cloning vector pBR322 cut with EcoRI and the ligation was transformed into DS.902, selecting on L-agar + Amp + Kan. The clone isolated thus was called pCS300 and is 19.5kb in size containing 4.316kb of pBR322 sequence, 5.77kb of Tn5 sequence and 9.3kb of flanking chromosomal DNA (Figure 5.1). Colin Stirling carried out a restriction analysis of this clone, to determine the position of Tn5. Tn5 was found to be centrally placed in the chromosomal DNA with 5.3kb to one side and 4kb to the other.

5.3 Cloning the wild type xerA gene.

The mutant clone pCS300 contains large regions of flanking chromosomal DNA to the left and right of Tn5 and so there was a good chance that this EcoRI fragment would contain a complete xer gene. An F' strain CGSC 4315 was available, which complements the Xer$^-$ phenotype of DSX.300, so it was decided to increase the probability of picking up the wild type xerA gene by using F' DNA. DNA of the strain DS.902, containing F'4315 was made using the cleared lysate method described in Materials and Methods. This DNA was subsequently subjected to two phenol extractions and two phenol chloroform extractions. The extract was then put through a CsCl/EtBr gradient, spinning for 16hrs at 49K. Two bands were visible in the UV light, the lower containing the F' DNA and the upper containing the chromosomal DNA. These bands were taken off separately and purified as described in Materials and Methods. The lower band contained the F' DNA and some chromosomal DNA and was used as the source of fragment DNA.
The cloning vector used was a ptac expression vector, based on pAT153, called pAT223 (Figure 5.2). This vector contains the tac promoter, which is a combination of the trp and lac promoters (de Boer et al, 1983). If this vector is transformed into a lacIq host, such as DS.941, then the tac promoter is repressed and may

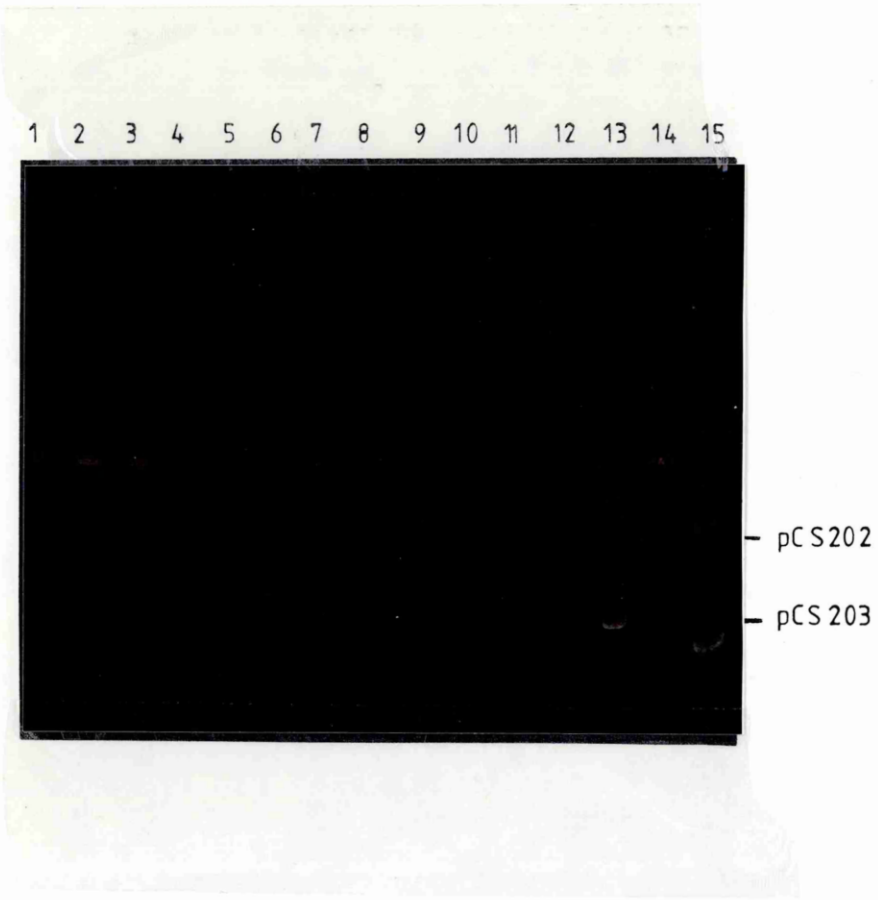


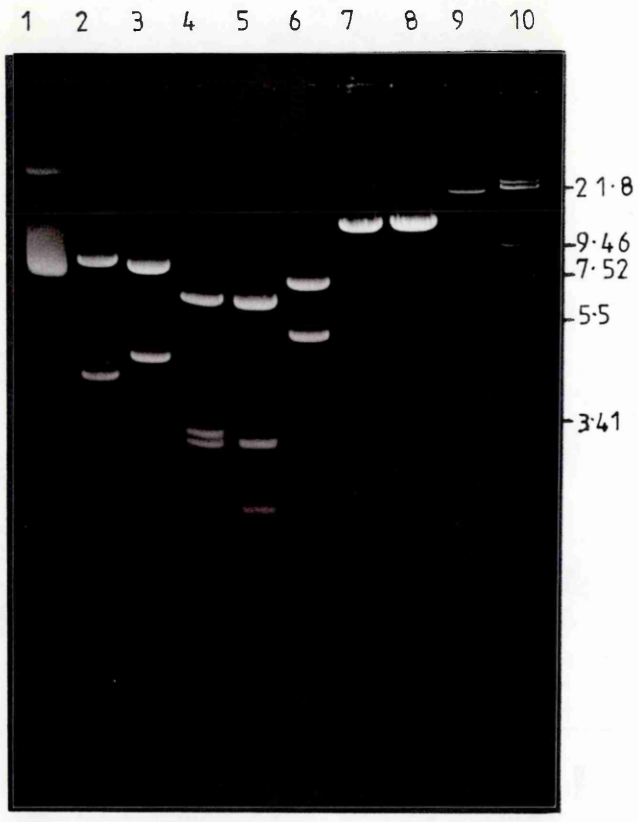
Figure 5.3 Complementation of the Xer$^-$ phenotype of DSX.330 with the wild type clone, pGS30.

Lanes (1-3) pCS202 + pGS30 in DSX.330
(4-6) pCS202 + pAT223 in DSX.330
(7-9) pCS202 + pGS30 in DSX.330
(10-11) pCS202 + pAT223 in DSX.330
(12) pCS202
(13) pCS203
(14) pGS30 (wild type clone)
(15) pAT223 (vector)

be derepressed at the appropriate time by the addition of IPTG. The tac promoter is followed by the pUC8 polylinker, facilitating the positioning of genes behind the promoter and ribosome binding site. Although it was decided to use an expression vector, it is possible that the xer gene will be expressed of its own promoter. The plasmid pAT223 contains a single site for the restriction endonucleases HindIII and EcoRI. The chromosomal insert in pCS300 also contains single sites for EcoRI and HindIII at the boundaries of the insert (Figure 5.1), which would give a wild type fragment of 8.45kb. It was decided to use HindIII and EcoRI as the cloning enzymes as this avoids the use of CIP, due to having heterologous ends. Hence, the vector pAT223 was cut with EcoRI and HindIII, the digest was run on a low melting point gel and the large vector fragment was cut out and purified. The fragment DNA was prepared from the F' enriched DNA. This F' DNA was cut with EcoRI and HindIII, and run on a 0.4% agarose gel, giving seven visible bands in the size range of 7-13kb. The portion of the gel containing these fragments was cut out and the DNA extracted by electrophoresis, as described in Materials and Methods. The fragment DNA was then precipitated and ligated to the purified HindIII, EcoRI cut pAT223, in an approximate ratio of three to one. Ligation was carried out overnight at 16° C. This ligation mix was transformed into the lacIq strain, DS.941. Single colony gels were used to screen for clones, initially picking up twelve of approximately the correct size, according to the size of the insert in the clone pCS300. DNA of these clones was made using the alkaline-SDS denaturation procedure (Birnboim and Doly, 1979), and transformed into DSX.330 containing the compatible pseudo-cer dimer, pCS202, to look for complementation. One clone was detected, which functionally complements the Xer$^-$ phenotype of DSX.330 (Figure 5.3). This clone was called pGS30 and contains 8.45kb of chromosomal DNA.

The wild type clone pGS30 was restriction mapped, using the

Figure 5.4A Restriction analysis of the clone, pGS30.



Lane (1) pGS30

(2) pGS30 digested with HindIII & EcoRI

(3) pGS30 digested with Bam HI

(4) pGS30 digested with SphI

(5) pGS30 digested with SphI & AccI

(6) pGS30 digested with AccI

(7) pGS30 digested with KpnI

(8) pGS30 digested with XhoI

(9) lambda digested with EcoRI

(10) lambda digested with HindIII

Plasmid	Enzyme used	Size of Frags (kb)	Size of plasmid (kb)
pGS30	HindIII EcoRI	3.8 + 8.5	12.3
pGS30	BamHI	4.2 + 8	12.2
pGS30	SphI	6.2 + 3.1 + 3.0	12.3
pGS30	SphI AccI	6.2 + 3.0 + 2.2 + 0.9	12.3
pGS30	AccI	7.2 + 5.0	12.2
pGS30	KpnI	12.3	12.3
pGS30	XhoI	12.3	12.3

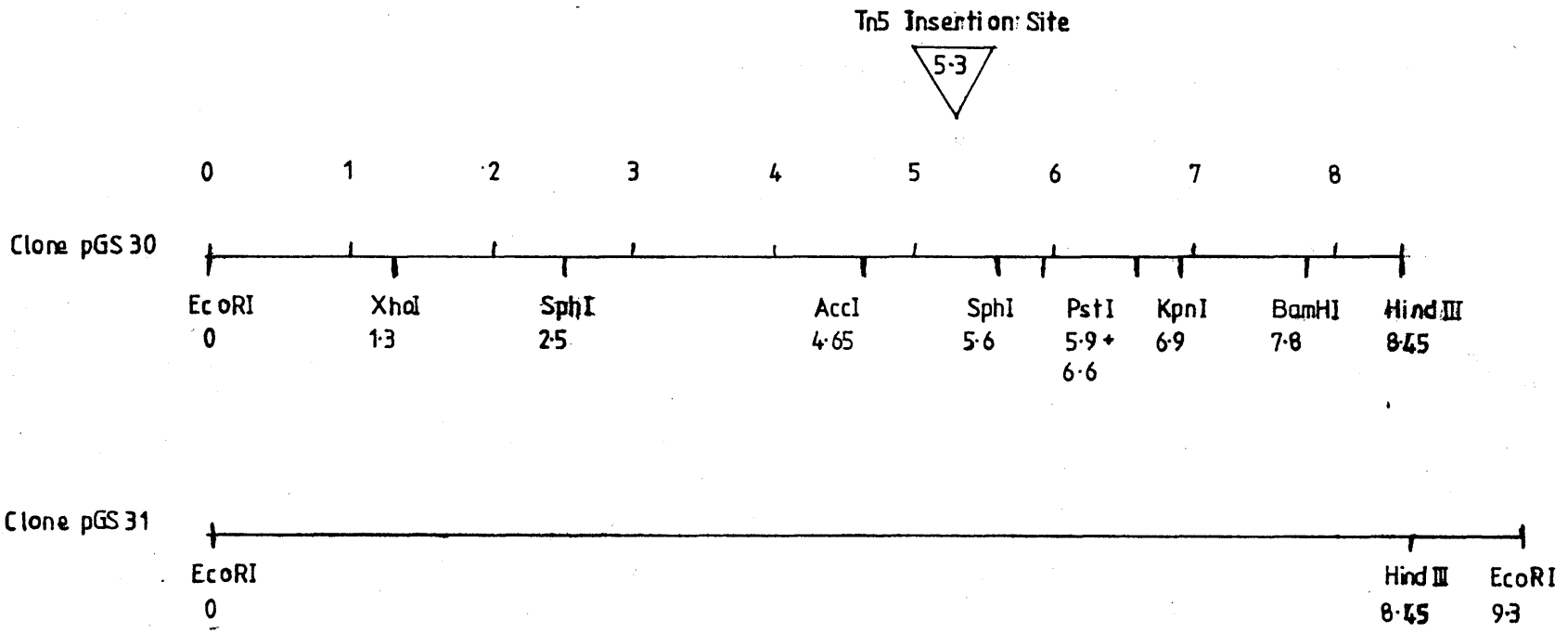


Figure 5.4B Restriction map of the chromosomal inserts in the clones pGS30 and pGS31.

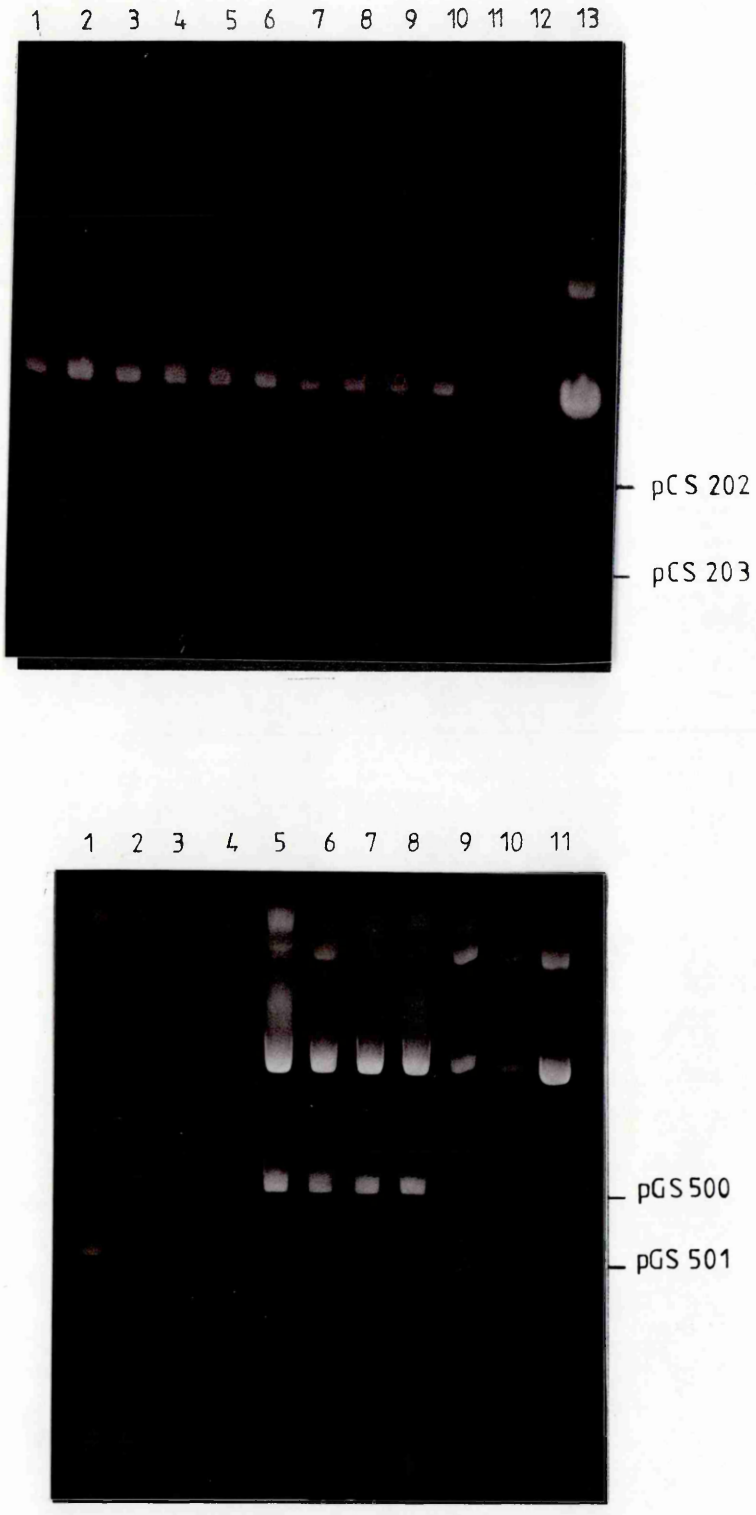


Figure 5.5 Single colony gels showing that the clone, pGS30 does not complement the Xer$^-$ phenotype of DSX.1 and 2.

Gel (A) - using the pseudo-cer test plasmid, pCS202.

Lanes (1-5) pCS202 + pGS30 in DSX.1

(6-10) pCS202 + pGS30 in DSX.2

(11) pCS203 in DS.903

(12) pCS202 in DSX.1

(13) pGS30

Gel (B) - using the pseudo-cer test plasmid, pGS500.

Lanes (1) pGS500 in DS.903

(2) pGS500 in DSX.1

(3) pGS500 in DSX.2

(4) pGS500 in DSX.330

(5-6) pGS500 + pGS30 in DSX.1

(7-8) pGS500 + pGS30 in DSX.2

(9-10) pGS500 + pGS30 in DSX.330

(11) pGS30

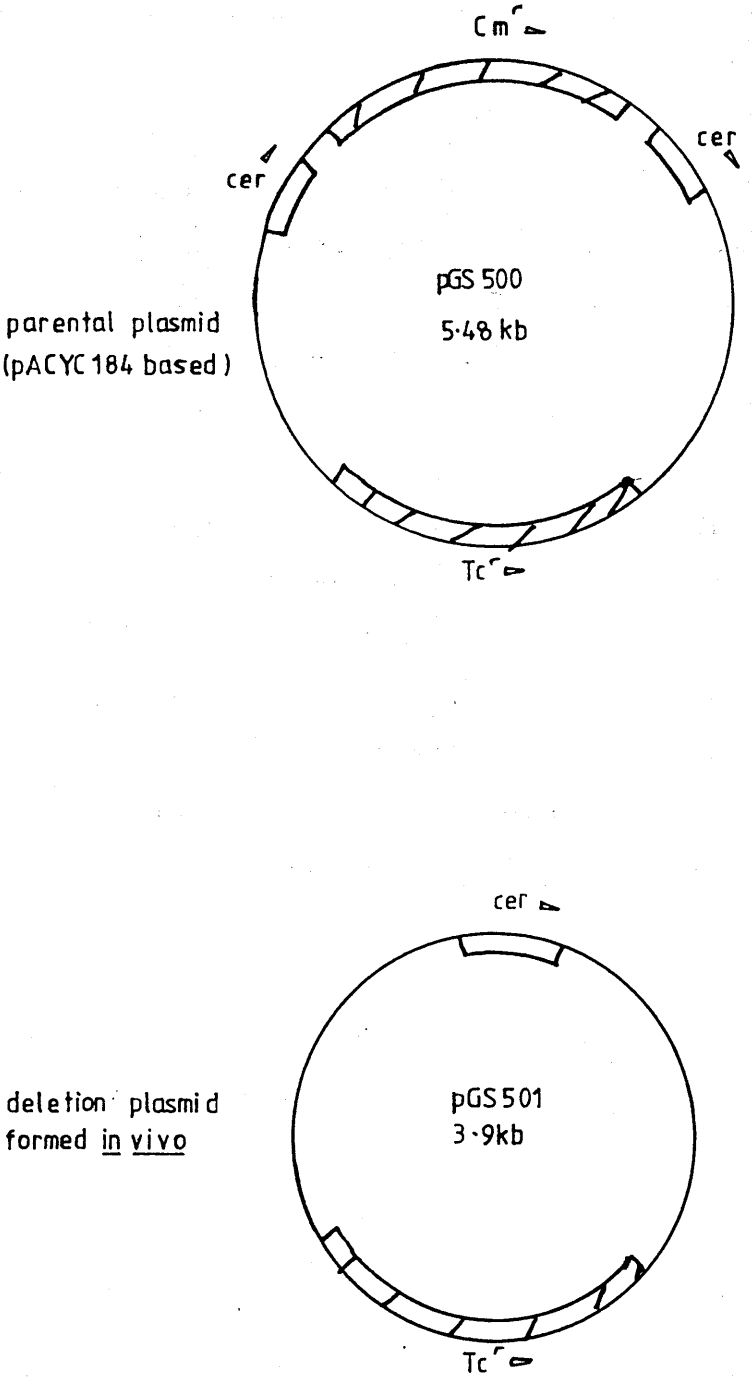


Figure 5.6 Organization of the pACYC184 based pseudo-cer dimer, called pGS500.

following restriction endonucleases: HindIII, EcoRI, BamHI, SphI, AccI, KpnI and XhoI (Figure 5.4A &B). The restriction map of the wild type clone, pGS30 was found to correspond to that of the mutant clone pCS300. The only difference being that the mutant clone, pCS300 contains Tn5, which the wild type clone, pGS30 does not. The mutant clone pCS300 contains a 0.85kb HindIII-EcoRI boundary fragment, which pGS30 does not, therefore pGS31 was constructed by cloning the 0.85kb fragment from pCS300 into pGS30 (Figure 5.4B).

5.4 Does the wild type clone, pGS30 functionally complement the mutant Xer phenotype of DSX.1 and DSX.2?

The clone pGS30 functionally complements the Xer$^-$ phenotype of DSX.330 but does it complement the mutant Xer phenotype of DSX.1 and 2. To determine this DSX.1 and DSX.2 were transformed with the pseudo-cer dimer, pCS202 and their Xer status confirmed on single colony gels. These strains were then transformed with the wild type clone, pGS30, selecting for transformants on L-agar + Cm + Amp and examined on single colony gels (Figure 5.5A). The clone pGS30 was found not to complement the Xer$^-$ phenotype of DSX.1 and 2. To confirm this complementation data another pseudo-cer dimer, pGS500 was used (Figure 5.6). DSX.330, DSX.1 and DSX.2 were transfomed with pGS500 and their Xer status confirmed on single colony gels. These strains were then transformed with pGS30, selecting on L-agar + Tc + Amp and examined on single colony gels (Figure 5.5B). Clone pGS30 was found to functionally complement DSX.330 but not DSX.1 and 2, confirming the data obtained with pCS202. The largest wild type clone pGS31 containing a 9.3kb insert does not functionally complement the Xer$^-$ phenotype of DSX.1 and 2 either.

Why does the wild type clone complement the Xer$^-$ phenotype of DSX.330 but not that of DSX.1 and 2? One possible explanation

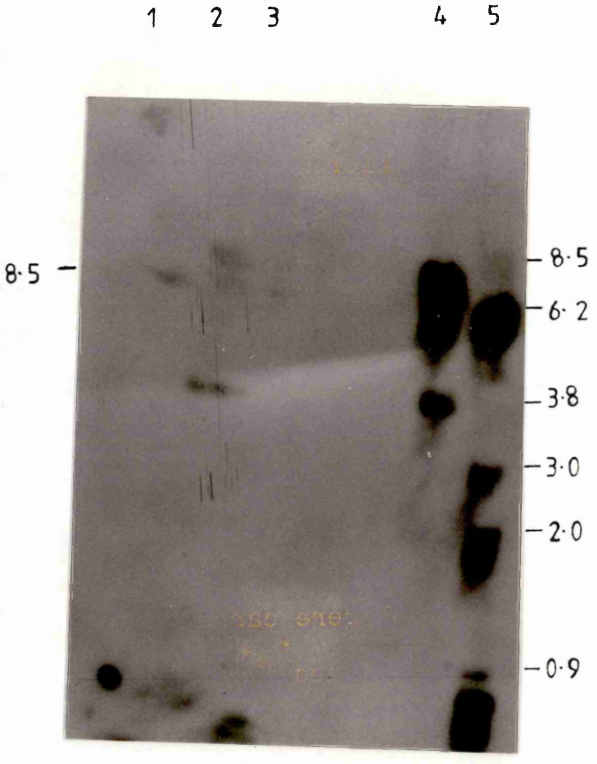


Figure 5.7 Southern blot analysis of DS.903, DSX.1 and DSX.2 probed with the clone pGS30, containing the wild type xerA gene.

Lane (1) DS.903 chromosomal DNA digested with EcoRI & HindIII

(2) DSX.1 chromosomal DNA digested with EcoRI & HindIII

(3) DSX.2 chromosomal DNA digested with EcoRI & HindIII

(4) pGS30 digested with EcoRI & HindIII

(5) pGS30 digested with AccI & SphI

Note that the probe pGS30 lights up an 8.5kb EcoRI-HindIII band, in DS.903, DSX.1 and DSX.2, as expected if the xerA gene contains no large deletions in these strains.

could be that complementation in DSX.330 is occurring via gene conversion but not in DSX.1 and 2 as these mutants have a deletion extending beyond at least one of the boundaries of the insert in the wild type clone. If this were so then one would not expect pGS30 to complement a recA$^-$ derivative of DSX.330. Alternatively using the clone pGS30 as a probe it should be possible to show a smaller band lighting up in DSX.1 and 2 than in the parent strain, DS.903. However this requires that DSX.1 and 2 are deletion mutants and that the deletion of the 8.45kb fragment is big enough to detect. With these provisos in mind the experiment was carried out. DNA of DS.903, DSX.1, DSX.2 and pGS30 were cut with EcoRI and HindIII, run on a 0.8% agarose gel and Southern blotted to pall membrane. This blot was then probed with nick translated pGS30. The prehybidization and hybridization conditions were carried out at 65^o C, washing was as described in Materials and Methods. The probe pGS30 lit up a band of 8.45kb in DS.903, DSX.1 and DSX.2 (Figure 5.7). This is consistent with DSX.1 and 2 not being large deletions of any part of the wild type clone, pGS30.

5.5.1 Construction of a recA$^-$ derivative of DSX.300.

The complementation data to date has been carried out in a recA$^+$, recF$^-$ background, hence the "complementation" that has been observed could be due to gene conversion. To eliminate this possibility a recA$^-$ version of DSX.300 was required. This was constructed from DS.902, which is a recA$^-$, xer$^+$ strain. In order to P1 transduce this strain to a Xer$^-$ phenotype, it had to be made temporarily recA$^+$. This was acheived by transforming DS.902 with ppE14, which is a pACYC184 based plasmid containing the recA gene. A P1 lysate made on DSX.300 was then used to P1 transduce DS.902 containing ppE14(Cm^r), selecting for Kan^r colonies. Three Kan^r transductants were selected. From previous

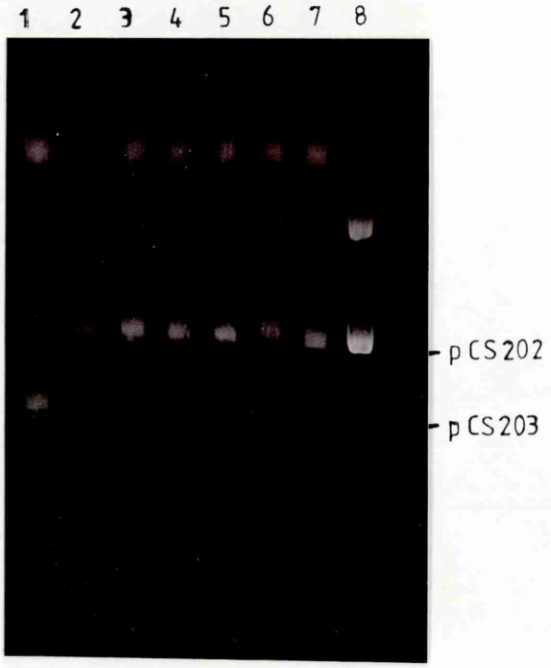


Figure 5.8 Confirmation of the Xer$^-$ phenotype of DSX.360, DSX.361 and DSX.362, in comparison to the parental Xer$^+$ strain DS.902, using the pseudo-*cer* test plasmid, pCS202.

Lanes (1) pCS202 in DS.902

(2-3) pCS202 in DSX.360

(4-5) pCS202 in DSX.361

(6-7) pCS202 in DSX.362

(8) pCS202

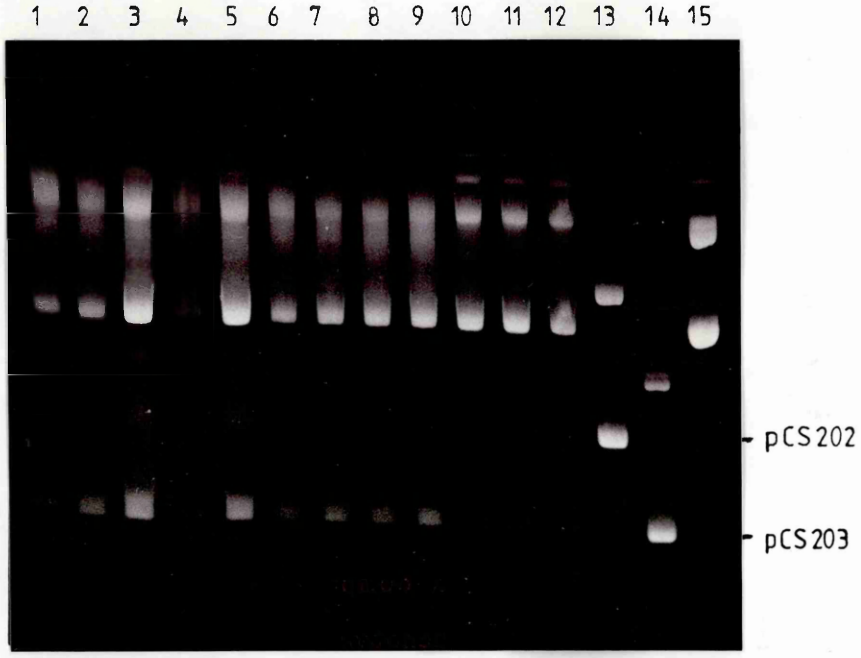


Figure 5.9 Complementation of the Xer⁻ phenotype of DSX.360, 361 and 362, with the wild type clone pGS30.

Lanes (1-3) pCS202 + pGS30 in DSX.360
(4-6) pCS202 + pGS30 in DSX.361
(7-9) pCS202 + pGS30 in DSX.362
(10-12) pCS202 + pGS30 in DSX.330
(13) pCS202
(14) pCS203
(15) pGS30

experience 100% of the transductants, which are Kan^r are also Xer$^-$ in phenotype. These three Kan^r transductants were then streaked on L-agar + crystal violet and restreaked through single colonies a number of times, to screen for segregants which have lost ppE14, and are therefore Cm^s. Eventually segregation of Cm$^{r/s}$ phenotypes occurred i.e. segregation of white/purple phenotypes on L-agar + crystal violet. The recA status of these isolates was then determined by UV sensitivity, as described in Materials and Methods. A number of isolates were recA$^+$ probably due to gene conversion, however three were obtained which were recA$^-$. These three isolates were transformed with the pseudo-cer dimer, pSC202, to test their Xer phenotype and examined on single colony gels (Figure 5.8). The recA$^-$ Kan^r P1 transductants are Xer$^-$ in phenotype in comparison to the parental strain DS.902, which is Xer$^+$ in phenotype. The new strains were called DSX.360, 361 and 362, and are recA$^-$, xer$^-$.

5.5.2 Does the clone pGS30 complement a recA$^-$ derivative of DSX.330?

The newly constructed strains, DSX.360, 361 and 362 are recA$^-$ xerA$^-$, therefore the only way in which complementation of the Xer$^-$ phenotype can occur is if the clone pGS30 provides in trans the wild type gene product, which is mutant in these strains.
The strains, DSX.360, 361, 362 and 330 containing pCS202 were transformed with pGS30, selecting for transformants on L-agar + Cm + Amp. These transformants were examined on single colony gels (Figure 5.9). The gel shows that the wild type clone, pGS30 complements the Xer$^-$ phenotype of DSX.360, 361 and 362 as well as it does that of DSX.330, the recA$^+$, recF$^-$ strain. From this it can be concluded that the clone pGS30 carries the wild type xer gene, whose product is capable of trans complementation of the Xer$^-$ phenotype, of DSX.360.

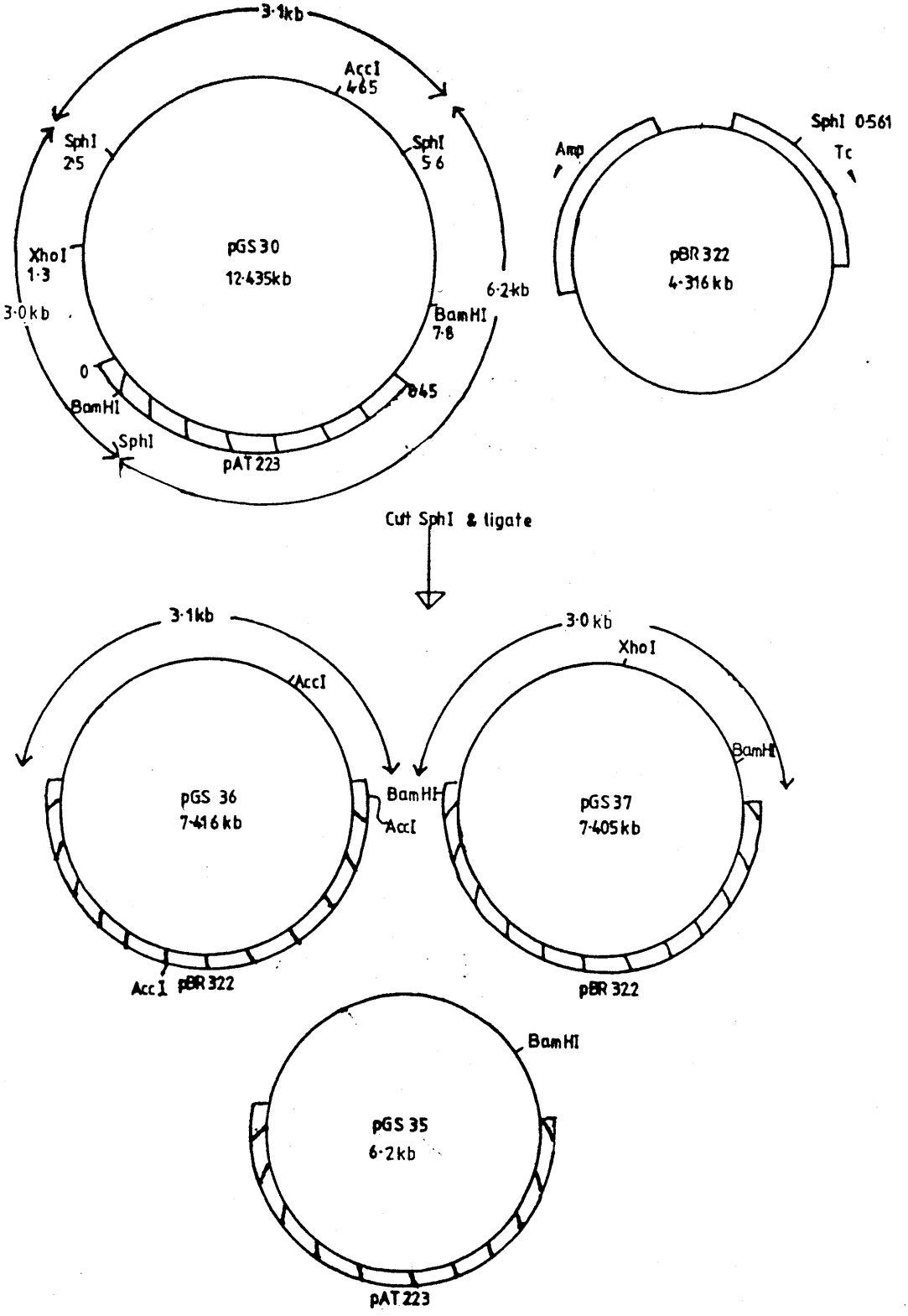


Figure 5.10 Sub-cloning of the SphI fragments of pGS30.

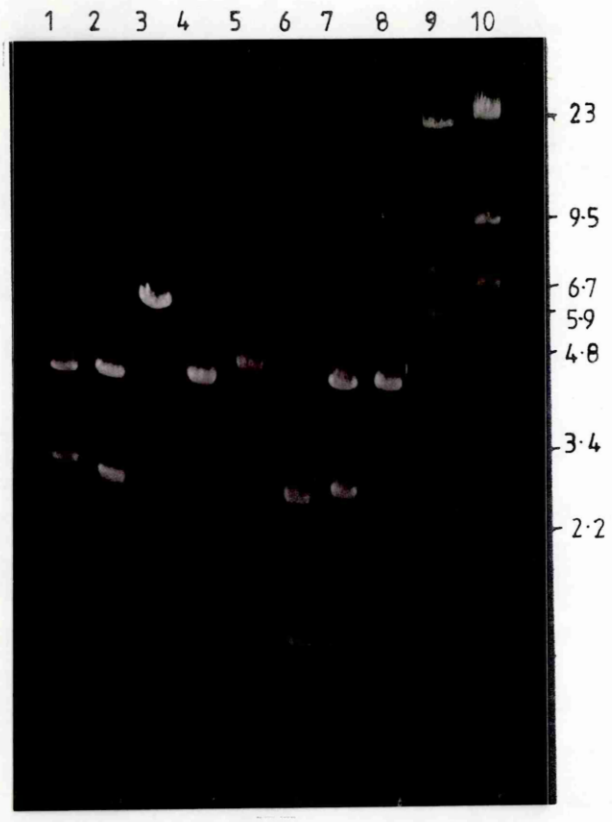


Figure 5.11 Restriction analysis of the clones pGS35, pGS36 and pGS37.

Lane (1) pGS36 digested with SphI

(2) pGS37 digested with SphI

(3) pGS35 digested with SphI

(4) pBR322 digested with SphI

(5) pGS36 digested with AccI

(6) pBR322 digested with AccI

(7) pGS37 digested with BamHI

(8) pBR322 digested with BamHI

(9) lambda digested with EcoRI

(10) lambda digested with HindIII

Plasmid	Enzyme used	Fragment sizes (kb)	Plasmid Size (kb)
pGS36	SphI	4.3 + 3.1	7.4
pGS37	SphI	4.3 + 3.0	7.3
pGS35	SphI	6.0	6.0
pBR322	SphI	4.3	4.3
pGS36	AccI	4.6 + 1.6 + 1.2	7.4
pBR322	AccI	2.7 + 1.6	4.3
pGS37	BamHI	4.3 + 2.8	7.1
pBR322	BamHI	4.3	4.3

The SphI digests confirm the size of the inserted fragments, and the AccI and BamHI digests show that the orientation of these fragments is as in figure 5.10.

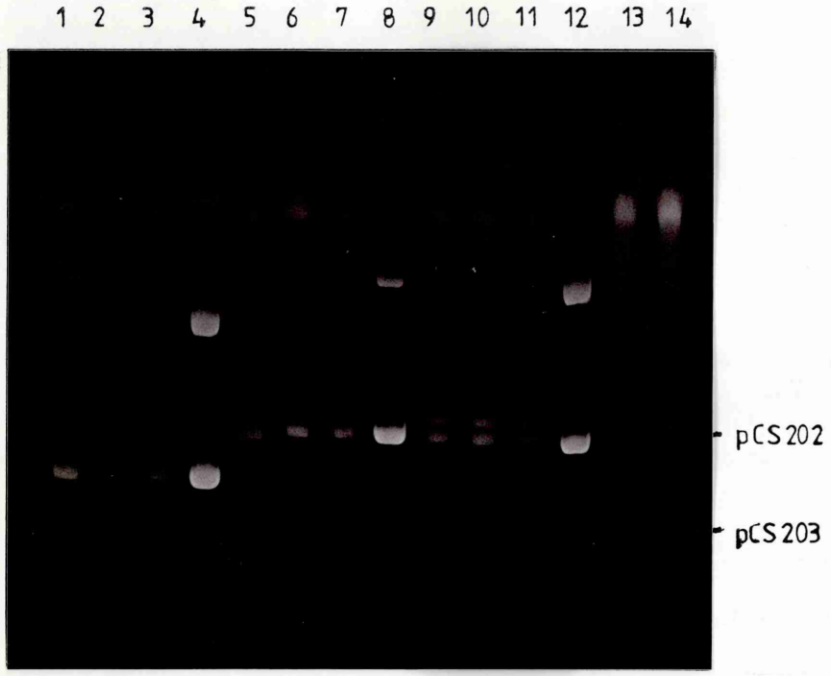


Figure 5.12 Testing the complementation of the Xer$^-$ phenotype of DSX.360 with the clones pGS35, pGS36 and pGS37.
Note that the clone pGS36 complements the Xer$^-$ phenotype of DSX.360 but not pGS35 or pGS37.

Lanes (1-3) pCS202 + pGS35 in DSX.360
(4) pGS35
(5-7) pCS202 + pGS36 in DSX.360
(8) pGS36
(9-11) pCS202 + pGS37 in DSX.360
(12) pGS37
(13) pCS203
(14) pCS202

5.6 Defining the minimum complementing fragment.

Initially the functional complementing clone contained insert DNA of 8.45kb in length. Obviously it was necessary to define the minimum complementing clone. One of the most important reasons for doing this was to minimize the amount of DNA sequencing necessary to define the gene.

5.6.1 SphI subcloning from pGS30.

There are three SphI sites in the wild type clone pGS30 (Figure 5.10), defining fragments of 6.2kb, 3.1kb and 3.0kb. The vector used to subclone into was pBR322, which has one SphI site at 560bp in the tetracycline gene. The vector was cut with SphI and then CIPed to minimise self ligation and the clone pGS30 was cut with SphI to give the fragment DNA. The fragment and vector DNA were ligated in the ratio of 3:1, overnight at 16^{o} C. The ligation mix was then transformed into DS.903 and the clones were screened for inserts by examination of the Amp^{r}, Tc^{s} clones. Three clones were obtained from this ligation pGS35, pGS36 and pGS37 (Figure 5.10): pGS35 is pAT223 DNA plus flanking DNA recircularised; pGS36 is pBR322 plus the 3.1kb SphI fragment and pGS37 is pBR322 plus the 3.0kb SphI fragment. The orientation of these fragments in pGS36 and pGS37 was determined using restriction endonucleases, which cut asymmetrically in the insert DNA, namely AccI and BamHI (Figure 5.11).

The clones pGS35, pGS36 and pGS37 were transformed into DSX.360 containing pCS202 selecting on L-agar + Amp + Cm and examined on single colony gels for complementation of the Xer^{-} phenotype (Figure 5.12). The clones pGS35 and pGS37 were found not to complement the Xer^{-} phenotype of DSX.360, whereas the clone pGS36 does. The clone pGS36 contains a 3.1kb SphI fragment, within which lies the point of Tn5 insertion in the mutant clone pCS300.

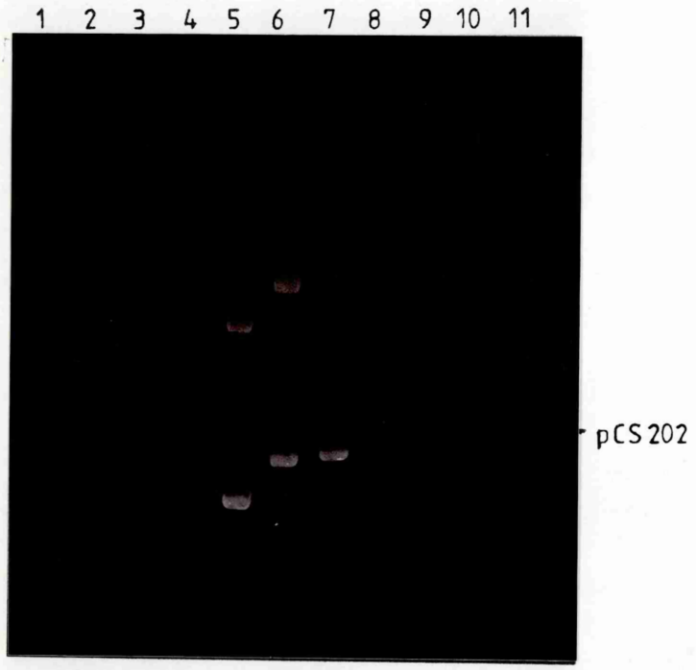


Figure 5.13 Single colony gel showing that the clones pGS35, pGS36 and pGS37 do not complement the Xer⁻ phenotype of DSX.1 and DSX.2.

Lane (1) pCS202 + pGS35 in DSX.1
(2) pCS202 + pGS37 in DSX.1
(3) pCS202 + pGS36 in DSX.1
(4) pCS202
(5) pGS35
(6) pGS37
(7) pGS36
(8) pCS202 + pGS35 in DSX.2
(9) pCS202 + pGS37 in DSX.2
(10) pCS202 + pGS36 in DSX.2
(11) pCS202

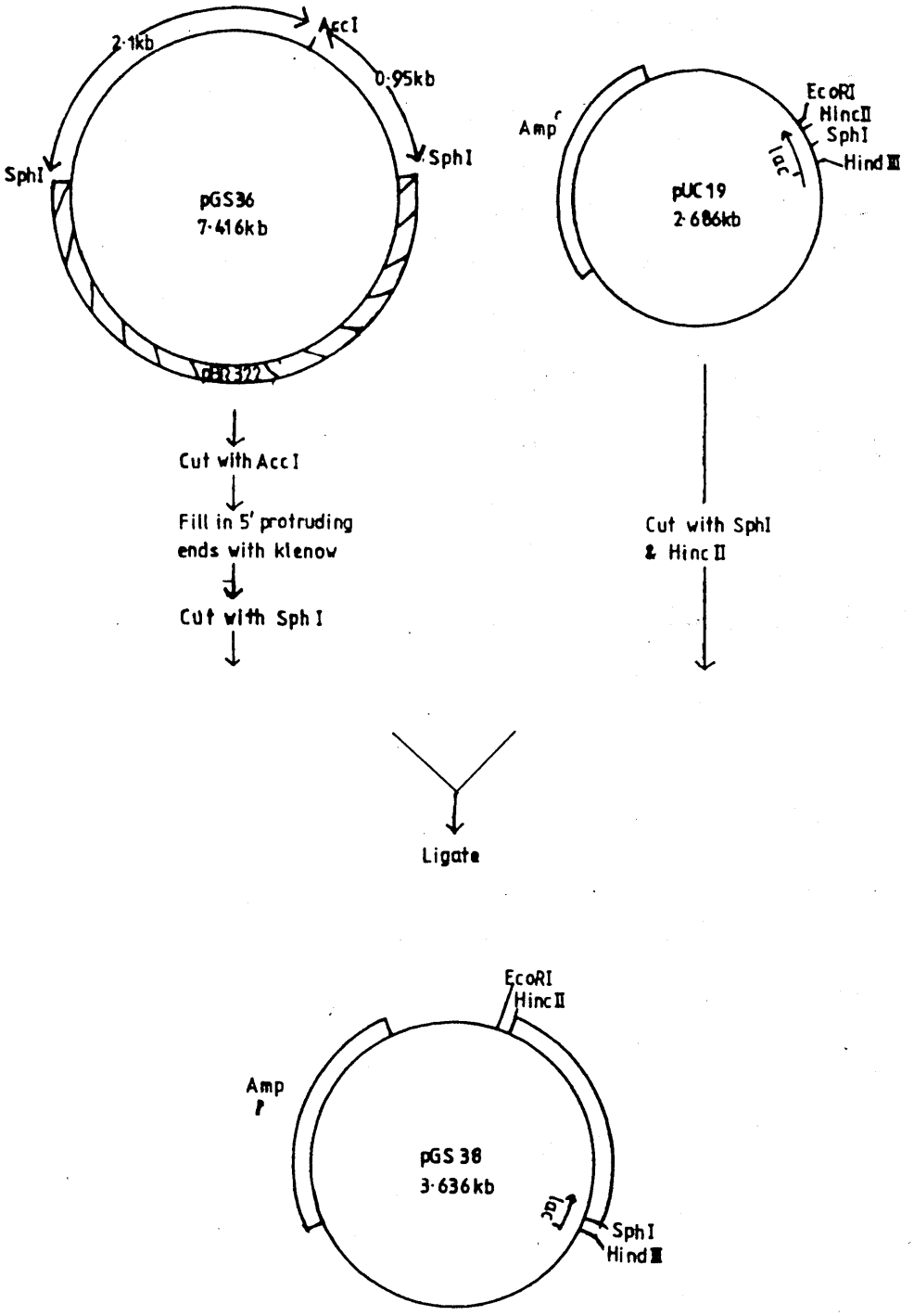


Figure 5.14A Sub-cloning of the 0.95kb AccI, SphI fragment from pGS36.

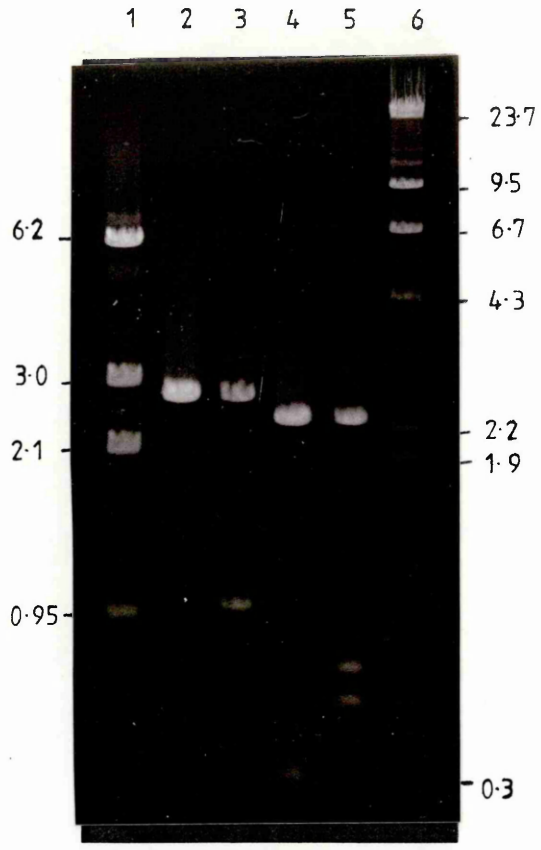


Figure 5.14B Restriction analysis of the clone pGS38.
Note that when the clone pGS38 is digested with EcoRI-HindIII, which bound the insert, then a 0.95kb band equivalent to that present in pGS30 is observed.

Lane (1) pGS30 digested with AccI & SphI
(2) pUC19 digested with EcoRI & HindIII
(3) pGS38 digested with EcoRI & HindIII
(4) pUC19 digested with PvuII
(5) pGS38 digested with PvuII
(6) lambda digested with HindIII

Plasmid	Enzymes used	Fragment Sizes(kb)	Plasmid Size(kb)
pGS30	AccI, SphI	6.2 + 3.0 + 2.1 + 0.95	12.3
pUC19	EcoRI, HindIII	2.7 + 0.030	2.73
pGS38	EcoRI, HindIII	2.7 + 0.95	3.65
pUC19	PvuII	2.4 + 0.3	2.7
pGS38	PvuII	2.4 + 0.75 + 0.5	3.65

These digests confirm the size of the insert in pGS38.

These subclones were also checked for complementation in DSX.1 and 2, containing pCS202. The clones pGS35, pGS36 and pGS37 were transformed into DSX.1 and 2, selecting on L-agar + Amp + Cm and examined on single colony gels (Figure 5.13). As was expected none of the smaller clones complement the Xer$^-$ phenotype of DSX.1 and 2.

5.6.2 AccI, SphI subcloning from pGS36.

At this stage the minimum complementing clone, pGS36, contained insert DNA of 3.1kb in length, could this complementing fragment be reduced further? It was decided to subclone from pGS36 an AccI, SphI fragment of 0.95kb in length (Figure 5.14A). This AccI, SphI fragment flanks the site of the Tn5 insertion in pCS300. To obtain the fragment DNA, clone pGS36 was digested with AccI, then the protruding 5' end was filled in with Klenow as described in Materials and Methods, and subsequently cut with SphI. The vector used for this subcloning was pUC19, which has unique cloning sites for SphI and HincII within the polylinker. The vector pUC19 was cut with SphI and HincII, then this digest was run on a low melting point gel and the larger vector fragment was cut out of the gel and purified. The fragment and vector DNA were mixed in the ratio of 4:1 and ligated overnight at 16^{o} C. The ligation mix was then transformed into DS.941, a lacIq strain, selecting on L-agar + Amp + X-gal. The clones were screened on single colony gels to pick out by size the clone containing an insert of 0.95kb. The clone was called pGS38 and was checked by cutting the fragment DNA back out of the polylinker (Figure 5.14B), using HindIII and EcoRI, which flank SphI and HincII in the polylinker. When pGS38 is cut with EcoRI and HindIII, two fragments can be visualised, one of 2.7kb and the other of 0.95kb This confirms that pGS38 contains the correct insert of 0.95kb. Can pGS38, containing the 0.95kb AccI,

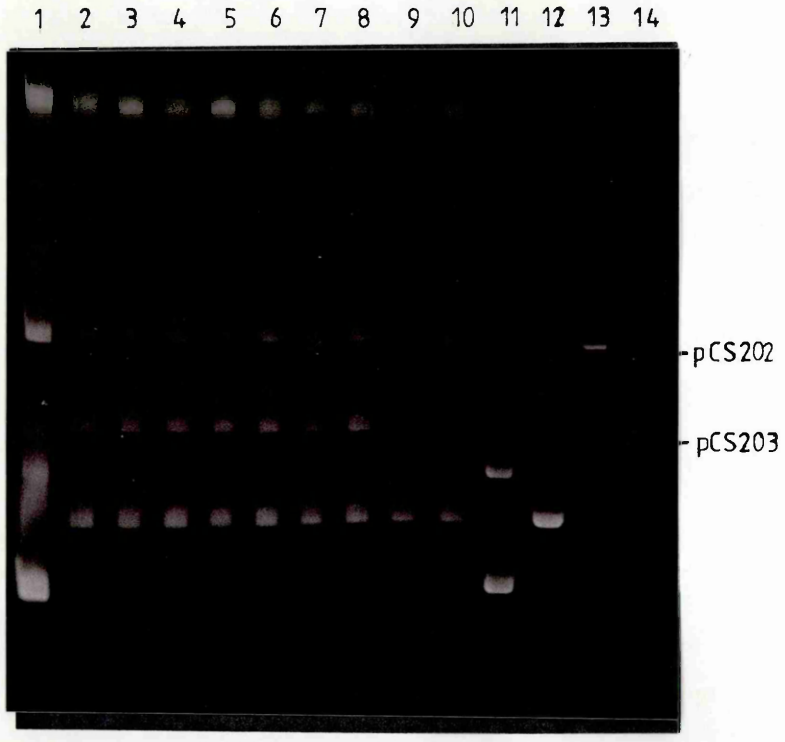


Figure 5.15 Complememtation of the Xer$^-$ phenotype of DSX.360 with pGS38.

Lanes (1) pCS202 + pUC19 in DSX.360
(2-8) pCS202 + pGS38 in DSX.360
(9-10) pCS202 + pGS38 in DSX.330
(11) pUC19
(12) pGS38
(13) pCS202
(14) pCS203

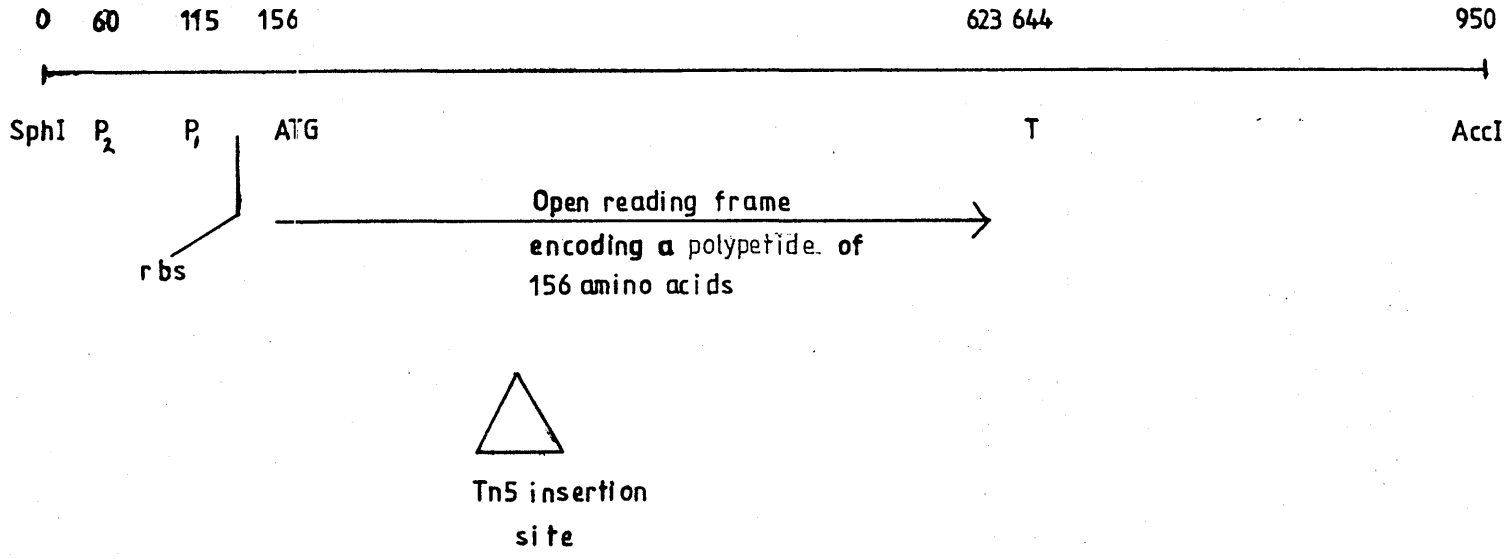
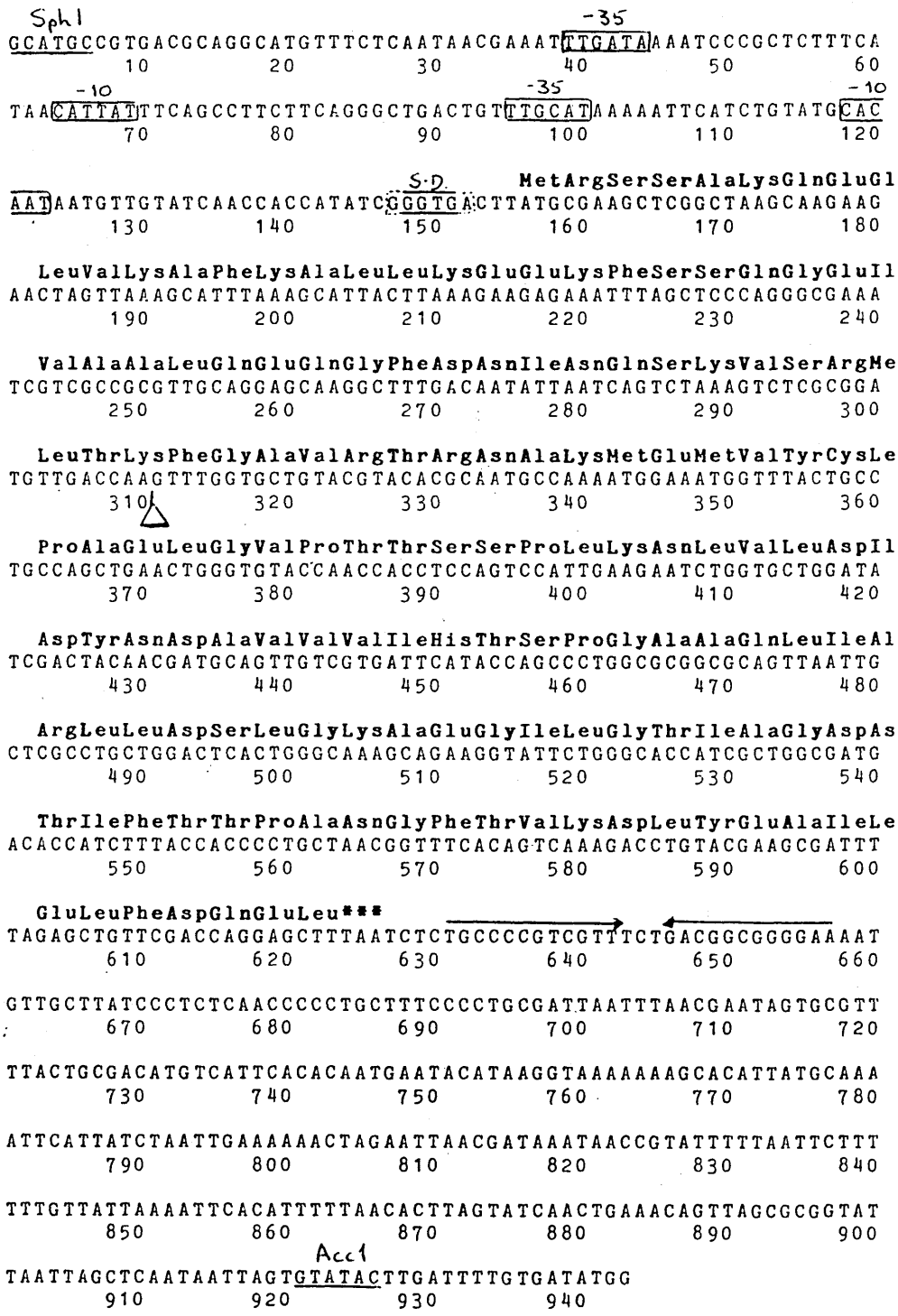


Figure 5.16A A map of the insert DNA of clone, pGS38 showing consensus promoters, rbs and terminators.
Note the position of the Tn5 insertion point in the mutant clone pCS300, disrupting the ORF.


Sph I
-35
-10
-35
-10
S·D.
MetArgSerSerAlaLysGlnGluGlu
LeuValLysAlaPheLysAlaLeuLeuLysGluGluLysPheSerSerGlnGlyGluIle
ValAlaAlaLeuGlnGluGlnGlyPheAspAsnIleAsnGlnSerLysValSerArgMet
LeuThrLysPheGlyAlaValArgThrArgAsnAlaLysMetGluMetValTyrCysLeu
ProAlaGluLeuGlyValProThrThrSerSerProLeuLysAsnLeuValLeuAspIle
AspTyrAsnAspAlaValValValIleHisThrSerProGlyAlaAlaGlnLeuIleAla
ArgLeuLeuAspSerLeuGlyLysAlaGluGlyIleLeuGlyThrIleAlaGlyAspAsp
ThrIlePheThrThrProAlaAsnGlyPheThrValLysAspLeuTyrGluAlaIleLeu
GluLeuPheAspGlnGluLeu***
Acc1

SphI fragment, complement the Xer⁻ phenotype of DSX.360. The clone pGS38 was transformed into DSX.360 containing pCS202, selecting on L-agar + Amp + Cm. These transformants were then examined on single colony gels (Figure 5.15). Clone pGS38 does functionally complement the Xer⁻ phenotype of DSX.360. At the first patching after transformation a mixture of parental and deletion pCS202 is observed, if these are then repatched and examined on single colony gels pCS202 is present in the deletion form.

Colin Stirling has sequenced this 0.95kb AccI, SphI fragment, one strand from the wild type and the other from the mutant clone pCS300, to obtain the point of Tn5 insertion (Figure 5.16). Computer analysis of this fragment reveals an ORF, which has two possible promoters, a good ribosome binding site and could encode for a polypeptide of 17kd (Figure 5.16). The codon usage within the ORF is non-random, a feature strongly correlated with coding sequence.

Clone, pGS38 containing the 0.95kb fragment appears to complement in DSX.360 but not as efficiently as the large wild type clone, pGS30, containing an 8.45kb fragment. This implies that there are some other sequences required for effective complementation. For example one could postulate that the xer gene is autoregulated by control sequences but when the gene is removed from these control sequences, as is the case in the clone pGS38, then the xer gene product is over or under-produced. This may have the effect of slowing down cer-specific recombination, which is the observation with the clone, pGS38. The observed affect is not due to the high copy number of pGS38, as when the AccI-SphI fragment is sub-cloned into a lower copy number vector the same affect occurs. If more time had been available sub-clones would have been constructed from the large clone pGS30, to test exactly which sequences are required for efficient cer-specific recombination.

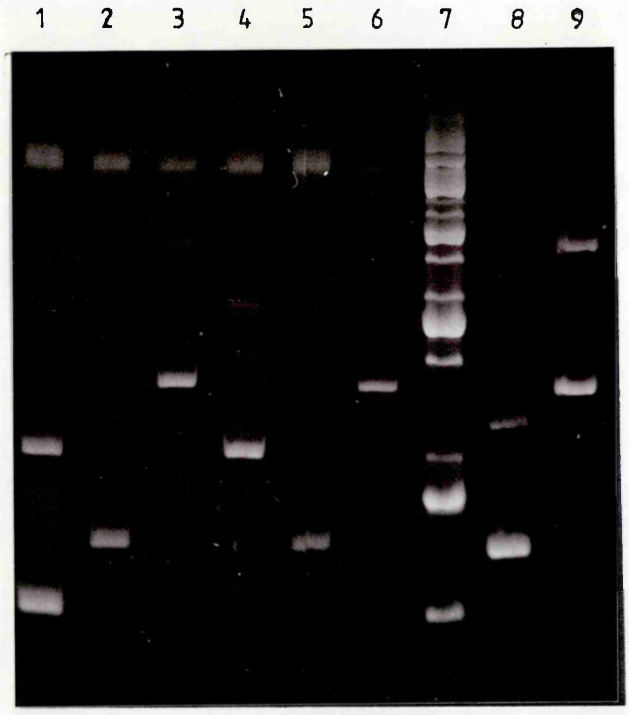


Figure 5.17 Single colony gel of the samples to be run on the whole cell protein gel.

Lane (1) pUC19 in DS.903

(2) pGS38 in DS.903

(3) pGS36 in DS.903

(4) pUC19 in DSX.330

(5) pGS38 in DSX.330

(6) pGS36 in DSX.330

(7) pUC19

(8) pGS38

(9) pGS36

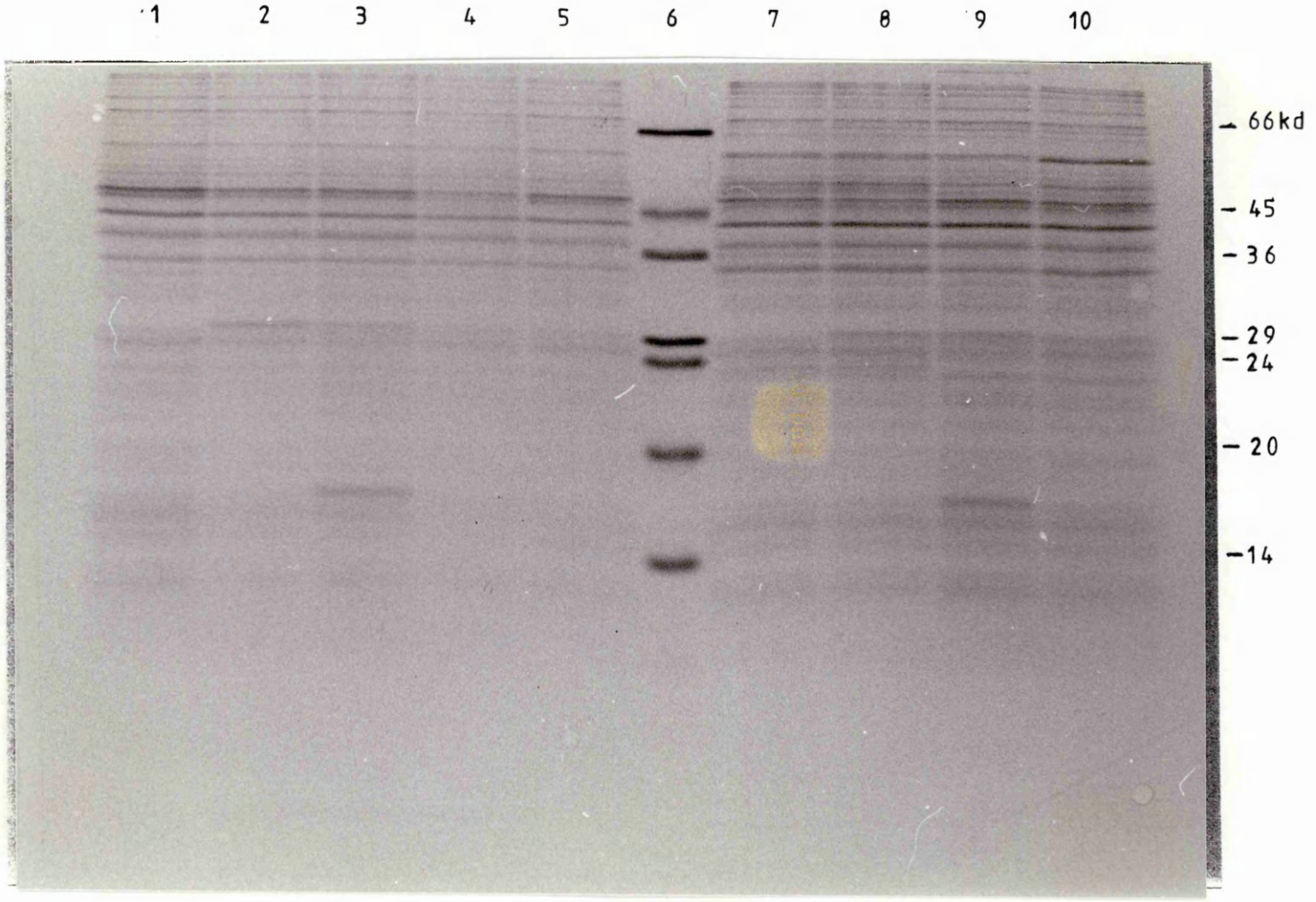

1
2
3
4
5
6
7
8
9
10
66kd
45
36
29
24
20
14

Figure 5.18 Whole cell protein extracts run on an 18% Laemmli gel.

Note the 17kd band in DS.903 and DSX.330 containing the plasmid, pGS38.

Lane (1) DS.903

(2) DS.903 + pUC19

(3) DS.903 + pGS38

(4) DS.903 + pGS36

(5) DS.903 + pBR322

(6) molecular weight markers

(7) DSX.330

(8) DSX.330 + pUC19

(9) DSX.330 + pGS38

(10) DSX.330 + pGS36

5.7 Are any insert specific polypeptides produced from the clones, pGS38 and pGS36?

A minimum complementing AccI-SphI fragment of 0.95kb has been defined. Colin Stirlings sequence analysis of this DNA fragment has shown the presence of an ORF which would encode for a polypeptide of 17kd, which has good consensus promoters and a ribosome binding site. Can any polypeptide specific to the inserts in pGS38 and pGS36 be visualised on Laemmli gels, using whole cell protein extracts?

DS.903 and DSX.330 were transformed with pUC19, pGS36 and pGS38. and checked out on single colony gels (Figure 5.17). Then the strains DS.903 and DSX.330 plasmid free and with the afore mentioned plasmids were grown-up overnight in L-broth plus appropriate drugs. 200ul of each of these overnight cultures were spun down and used to look at whole cell protein as desribed in Materials and Methods. The samples were run on an 18% Laemmli gel with a stacking gel of 4.5%. The gel was run slowly overnight at 10mAmps, stained with coomaisse blue and subsequently destained in methanol/acetic acid (Figure 5.18). A polypeptide was visible in the tracks containing pGS38, which was not present in the other tracks, containing just the strain or the strain plus pUC19. This polypeptide was approximately 17kd in size, which corresponds to the size of the ORF defined by sequence analysis of this 0.95kb AccI-SphI fragment. This 17kd polypeptide was not visible in the pBR322 clone, pGS36 but this may be because pBR322 has a much lower copy number than pUC19 or that in pGS36 the xerA gene product is not produced in such large amounts due to regulation.

To confirm that the 17kd polypeptide produced from pGS38 was insert-specific, this clone and pUC19 were run through the minicell protocol by Mark Rodgers. The plasmids pGS38 and pUC19 were transformed into DS.944, which is an AB1157, lacIq, minicell

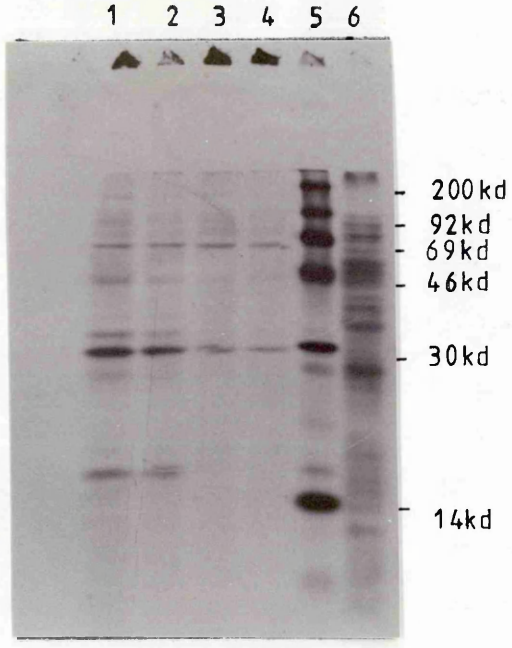


Figure 5.19 Examination of the clone pGS38 in the minicell strain, DS.944.

Lane (1) DS.944 + pGS38 + IPTG

(2) DS.944 + pGS38

(3) DS.944 + pUC19 + IPTG

(4) DS.944 + pUC19

(5) Molecular weight markers

(6) DS.944

Note the 17kd and 34kd bands are specific to the clone pGS38. The 34kd band could represent a dimer of the 17kd polypeptide.

strain; selecting on L-agar + Amp. These cells were grown up overnight in a rich growth media. The minicells were harvested on sucrose gradients and pulse labelled with [^{35}S] methionine. These cells were broken open and the protein extracts were examined on a 15% Laemmli gel (Figure 5.19). The autoradiograph of this gel showed two main polypeptides being expressed from pGS38, one being B-lactamase (29kd) (Dougan et al,1979), which was also produced from pUC19; the other polypeptide was produced only from pGS38 and is approximately 17kd in size. This confirms that the 17kd polypeptide seen on the whole cell Laemmli gels is in fact of plasmid origin and from the insert in pGS38.

Discussion and conclusions.

Colin Stirling derived a xer mutant, DSX.300 using λ467 (Kleckner ,1977). In P1 transduction experiments Tn5 and the mutant Xer phenotype were co-transduced at a high frequency implying that they are closely linked. This gave us the confidence to clone from DSX.300 the Tn5 sequences in the hope of selecting an xer gene. Tn5 has two useful properties, which allow cloning of this sequence, firstly that Tn5 carries antibiotic resistance genes, including Kan^r; and secondly that a number of restriction endonucleases do not cut within Tn5, including EcoRI. This allowed selection of a clone containing Tn5 plus flanking DNA from DSX.300. The clone was called pCS300 and contains 9.3kb of flanking DNA with Tn5 positioned centrally within it. As Tn5 has such a central position in the chromosomal DNA it was decided that there was a strong possibility that this EcoRI fragment would contain the xer gene mutant in DSX.300.

This allowed cloning of the analogous wild type fragment from a complementing F'. The clone isolated thus was called pGS30 and functionally complements the original xer$^-$, strain DSX.300. The strain DSX.300 is recA$^+$, which means that the complementation

that was observed may not necessarily be complementation but could be gene conversion. To eliminate this possibility a recA⁻ version of DSX.300 was constructed, called DSX.360. The wild type clone, pGS30 also complements a recA⁻ version of DSX.300, therefore it can be concluded that the clone pGS30 produces a product which can complement in trans the Xer⁻ phenotype of DSX.300.

The largest wild type clone, pGS31 containing 9.3kb of insert DNA, does not functionally complement the Xer⁻ phenotype of DSX.1 and 2. This implies that there are at least two host complementation groups required for cer-specific recombination.

The initial wild type clone, pGS30 contains a complementing fragment of 8.45kb in length. To define the gene it was necessary to subclone fragments of this 8.45kb and to test them for functional complementation. The first subcloning used SphI, which gives three fragments from pGS30. These three fragments were cloned into pBR322 and tested for complementation, only one clone was found to complement, which was pGS36, containing a 3.1kb SphI fragment bounding the Tn5 insertion point. The second subcloning used AccI and SphI to split the 3.1kb fragment into two fragments of 0.95kb and 2.15kb. The clone pGS38 containing the smaller AccI, SphI fragment of 0.95kb complements the Xer⁻ phenotype of DSX.360. The Tn5 insertion point is within this fragment. This defined the minimum fragment for sequencing. Computer analysis of the 0.95kb fragment reveals an ORF, encoding for a polypeptide of 17kd, which has a good consensus ribosome binding site, two alternative promoters and a terminator. A polypeptide of approximately 17kd was visualized on whole cell Laemmli protein gels and was confirmed as a genuine pGS38 insert-specific polypeptide using minicells.

In conclusion the minimum complementing fragment, which complements in trans the function mutant in DSX.360 is 0.95kb in length. The 0.95kb complementing fragment has an ORF encoding

for a polypeptide of 17kd, whose presence has been confirmed by examination of whole cell proteins and using minicells. The clone pGS31 containing the maximum complementing fragment of 9.3kb does not complement the Xer$^-$ phenotype of DSX.1 and 2, defining at least two host complementation groups necessary for cer-specific recombination.

The ColE1 cer site-specifc recombination system promotes intramolecular deletions between directly repeated cer sites, although inversions have been detected (S. Yaish, 1985). The reaction requirements of the cer system are as follows:

(i) two sites preferably in direct repeat. Summers et al (1985) have shown that the crossover occurs within 35bp but experiments of Summers (described in Chapter 1) show that a minimal fragment of 240bp is required. This implies that regions other than the crossover are necessary.

(ii) at least two host functions, one of which is encoded by the xerA gene. The roles of these host functions are as yet unknown, although one is probably the recombinase, that acts at cer. The other host factor could play a role in forming higher order protein-DNA structures or in DNA bending. The exact requirements of this system will only be determined when this ststem is set up in vitro, as has been for other site-specific recombination systems, such as the lambda integration system (Nash, 1975; Nash and Robertson, 1981).

The requirements of the cer-specific recombination system appear to be quite complex in comparison to some site-specific recombination systems. For example the loxP-cre system of the bacteriophage P1 is relatively simple, requiring two 34bp sites (loxP) and a single polypeptide recombinase, encoded by the cre gene (Hoess et al, 1982; Abremski and Hoess, 1984). A system with more stringent requirements is the site-specific recombination system of bacteriophage lambda. It requires a complex 240bp site (attP) and a 25bp site (attB), the integrase

(int) and another protein component integration host factor, which consists of two host polypeptides encoded by the himA and himD genes (Nash, 1975; Flamm and Weisberg, 1985; Kikuchi et al, 1985; Miller and Nash, 1981). The requirements of the inversion systems of Salmonella H-loop system (Zieg et al,1977), phage Mu G-loop system (Kamp et al,1979), P1 C-loop system (Iida et al, 1982) and the e14 defective prophage -pin- (Plasterk et al, 1983) are less well characterised. All of these systems require a recombinase, the product of the hin, gin, cin and pin genes and two inverted sites. In addition a requirement for host factors has been detetected in the G-loop system -FIS- (Johnson and Simon, 1985) and the H-loop system - FactorII and HU (Johnson et al, 1986). Some of the recombinases used in these different systems share sequence homology, for example 40% amino acid sequence homology has been demonstrated between hin and tnpR (Simon et al, 1980). On the other hand there are profound differences in the substrate specificity, in the additional factors required and in the nature of the reaction mediated by these site-specific recombination systems.

CHAPTER 6

PRELIMINARY MAPPING OF THE XER MUTANTS.

6.1 Introduction

The plasmid clone, pGS30 contains the wild type *xerA* gene, which is mutant in DSX.300. This clone can complement in *trans* the Xer$^-$ phenotype of DSX.300, but does not complement the Xer$^-$ phenotype of DSX.1 and DSX.2. These data imply that there are at least two host complementation groups required for *cer*-specific recombination. Where do these *xer* genes map to on the *E.coli* K-12 genetic map, do they form an operon or are they distant to each other? These questions can be answered using classical genetic techniques.

6.2 Mapping strategy

Two techniques have been employed to genetically map the *xer* genes:-

(i) Interrupted matings (Miller, 1972) - A donor Hfr strain and *xer*$^-$ recipient were mixed together, at different time intervals samples were taken and the mating pairs disrupted by agitation. The samples removed are diluted and plated out on media selecting for the recipient and for transfer of markers from the donor. These matings were used to determine a time of entry for the *xer* genes. It is more accurate when determining a time of entry that the marker under study enters within the first 30 mins, hence the reason for choice of Hfr strains. These Hfr matings were also used to carry out recombinational mapping for the genes *xer*1/2 and *xerA*. No direct selection procedure exsists for a Xer$^+$ phenotype, therefore exconjugants selected for transfer of an early marker were screened to determine their Xer phenotype using a pseudo-*cer* test plasmid.

(ii) F prime complementation - F's can be used to carry out *trans* complementation tests as they carry a defined region of the bacterial genome. When an F' is mobilized into a recipient

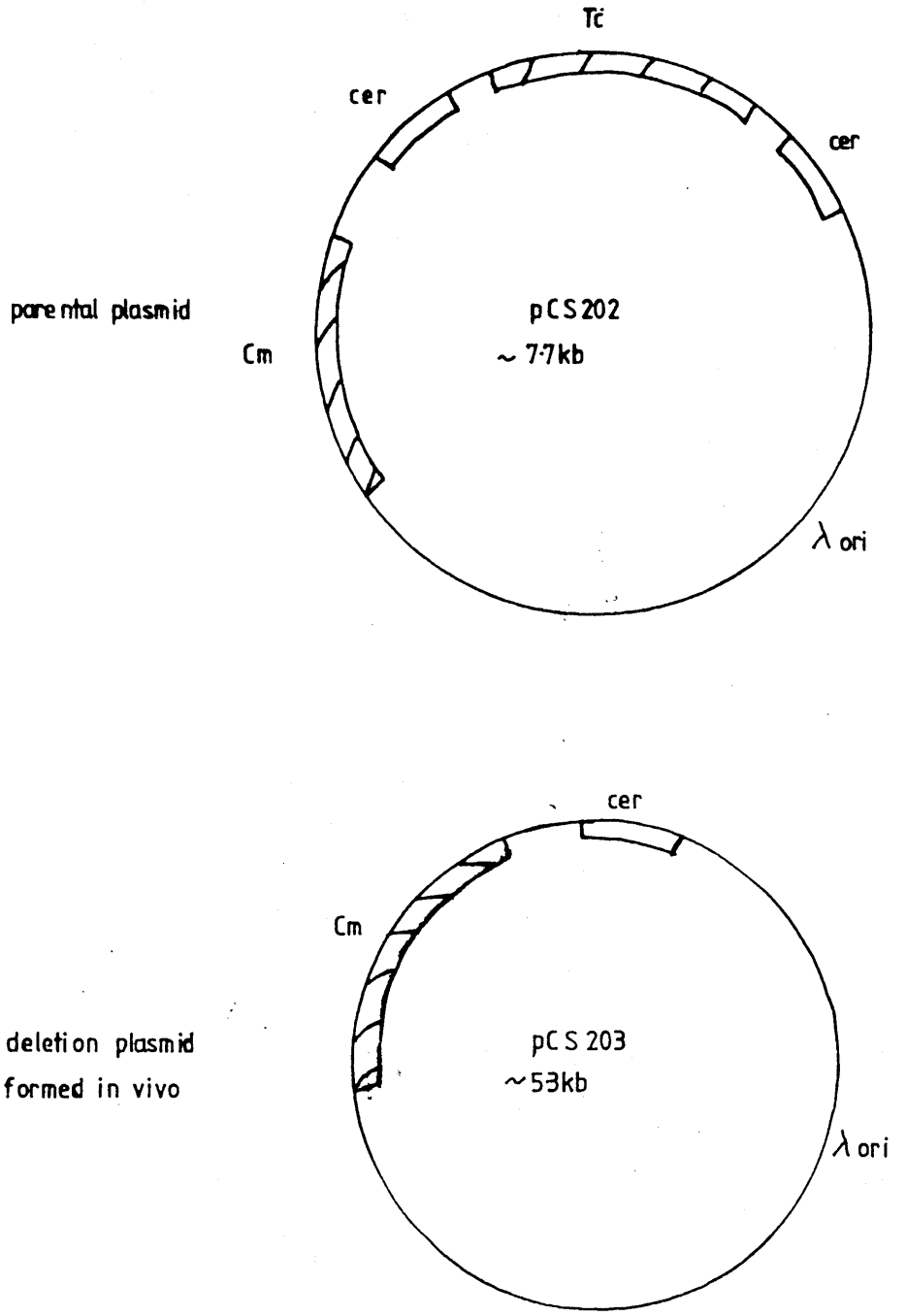


Figure 6.1 Organization of the λ-DV pseudo-cer test plasmid, pCS202.

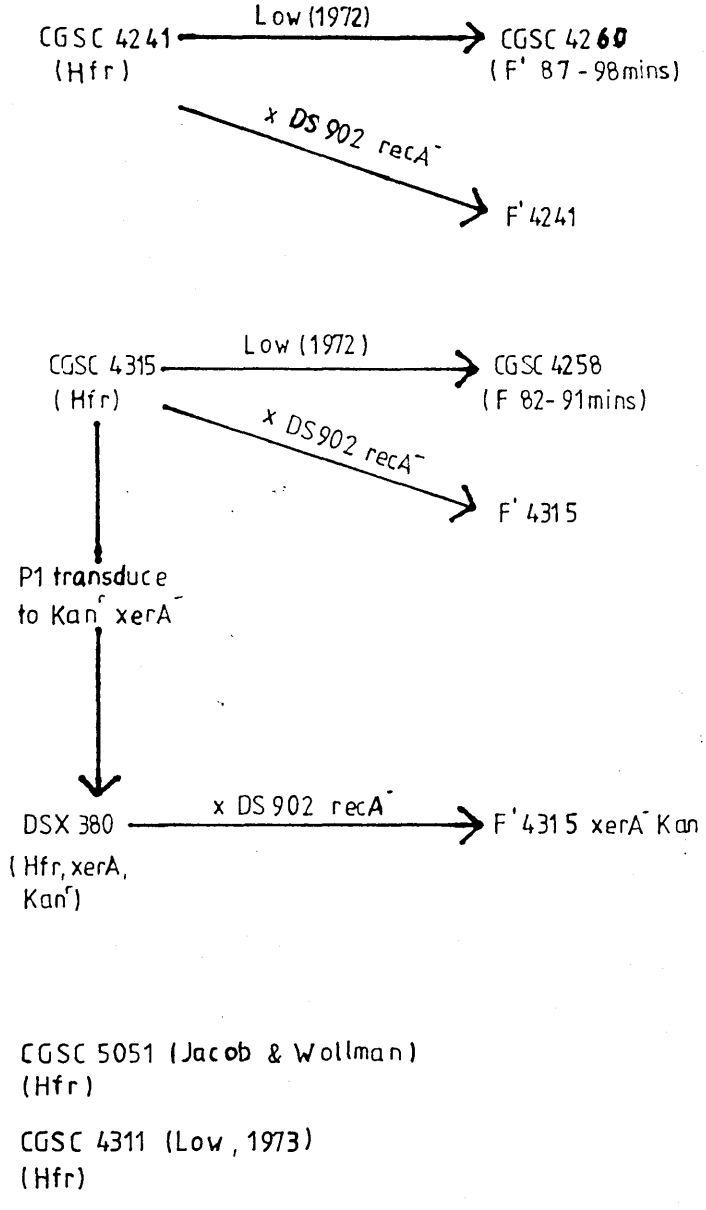


Figure 6.2 A summary of the Hfrs and F's used in the mapping strategy, illustrating their relationships to each other.

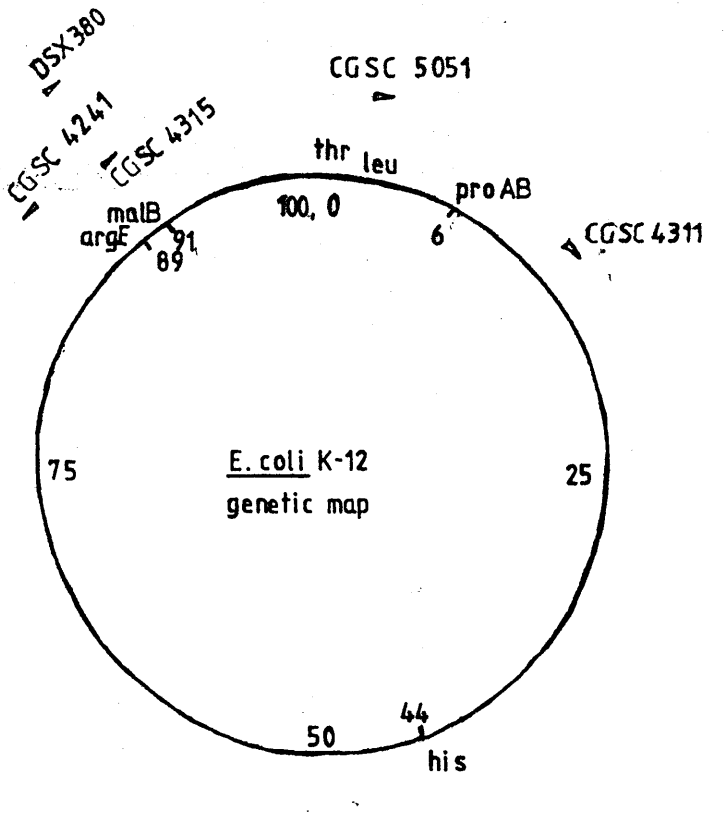


Figure 6.3 Illustration of the point of origin and direction of transfer of the Hfrs used.

Figure 6.4 Illustration of the instability of CGSC 4315

CGSC 4315 x DSX.1

No. of exconjugants (/ml)

Time(mins)	a^+	xyl^+	a^+ xer^+/tot
0	$3x10^3$	$1.5x10^3$	44/75
10	$1.6x10^4$	$1.9x10^3$	10/76
20	$4.0x10^4$	$3.7x10^3$	2/75
30	$8.1x10^4$	$4.9x10^3$	2/75
40	$1.9x10^5$	$2.4x10^4$	0/50
60	$3.7x10^5$	$7.7x10^4$	0/50

In this cross exconjugants selected for transfer of an early marker argE, were screened for Xer phenotype. At early time points a high proportion of the Arg^+ exconjugants were Xer^+ but this proportion decreased with time. This data implies that an F' carrying the wild type xer1 gene was being transfered early but this is being swamped at later time points by Hfr recombinants. The fact that the recombinants are not Xer^+ may imply that either xer1 lies just to the other side of the origin of this Hfr or close to the origin so that there is insufficient DNA for recombination to occur.

strain, that part of the bacterial genome which the F' carries is now present in duplicate. Appropriate F primes were used to confirm the time of entry map positions. F' matings were carried out on solid media, as described in Materials and Methods.

A set of Hfrs and F primes covering the entire E.coli K-12 chromosome were supplied courtesy of Barbara Bachmann.

In the crosses presented here the xer$^-$ recipient contained the pseudo-cer dimer, pCS202 to assess Xer phenotype (Figure 6.1). In a xer$^-$ exconjugant pCS202 would confer Cm^r and Tc^r, whereas in a xer$^+$ exconjugant pCS203 would confer Cm^r and Tc^s. Unless otherwise stated Naladixic acid was used as the male contra selection and to inhibit conjugal transfer after plating (Bouck and Adelberg, 1972).

A summary of the Hfrs and F's used to map the xer genes is presented in Figure 6.2. This figure shows the relationship between different Hfrs and F's.

6.3 Time of entry data of the xer genes mutant in DSX.1, DSX.2 and DSX.330.

Preliminary mapping data indicated that the xer genes lie between 80 and 90mins. Within this region lies the origin of two Hfr strains, which transfer DNA in opposite directions (Figure 6.3), CGSC4241 (Low, 1973) and CGSC4315 (Low, 1973). However initial experiments (Figure 6.4), indicated that these Hfrs were unstable, forming F's. Therefore an Hfr strain, CGSC 5051 (Jacob and Wollman) was used to derive a time of entry for the xer genes mutant in DSX.1, DSX.2 and DSX.330. This Hfr strain has its point of origin at 2.5 mins and transfers DNA in a clockwise direction to the recipient (Figure 6.3). The data for the crosses:

CGSC 5051 x DSX.1 Nal^r + pCS202

CGSC 5051 x DSX.2 Nal^r + pCS202

Figure 6.5 Data for the crosses CGSC 5051 x DSX.1, DSX.2, DSX.330 and DS.903.

DSX.1

No of exconjugants(/ml)

Time(mins)	tl^+	a^+	tl^+ xer^+/tot
0	1.3×10^3	0	0/50
10	1.5×10^3	0	0/50
20	2.7×10^3	2×10^1	4/50
30	5.4×10^3	7×10^1	10/50
40	1.0×10^4	2.5×10^2	17/50
60	2.6×10^4	2.9×10^3	40/100

plotted on graph 1

DSX.2

No of exconjugants(/ml)

Time(mins)	tl^+	a^+	tl^+ xer^+/tot
0	1.0×10^3	0	0/50
10	1.2×10^3	0	0/50
20	3.3×10^3	2×10^1	2/50
30	7.8×10^3	9×10^1	13/50
40	2.0×10^4	4.6×10^2	11/40
60	4.1×10^4	9.2×10^3	51/100

plotted on graph 2

DSX.330

No of exconjugants(/ml)

Time(mins)	tl^+	a^+	tl^+ xer^+/tot	a^+ xer^+/tot
0	1.4×10^3	0	0/25	-
10	1.6×10^3	2.0×10^1	0/25	N.T.
20	2.3×10^3	1.3×10^2	1/25	3/9
30	2.5×10^3	4.0×10^2	5/25	5/12
40	4.5×10^3	5.3×10^2	10/25	12/20
60	1.1×10^4	1.1×10^3	12/25	16/50

plotted on graph 4

DS.903

No of exconjugants(/ml)

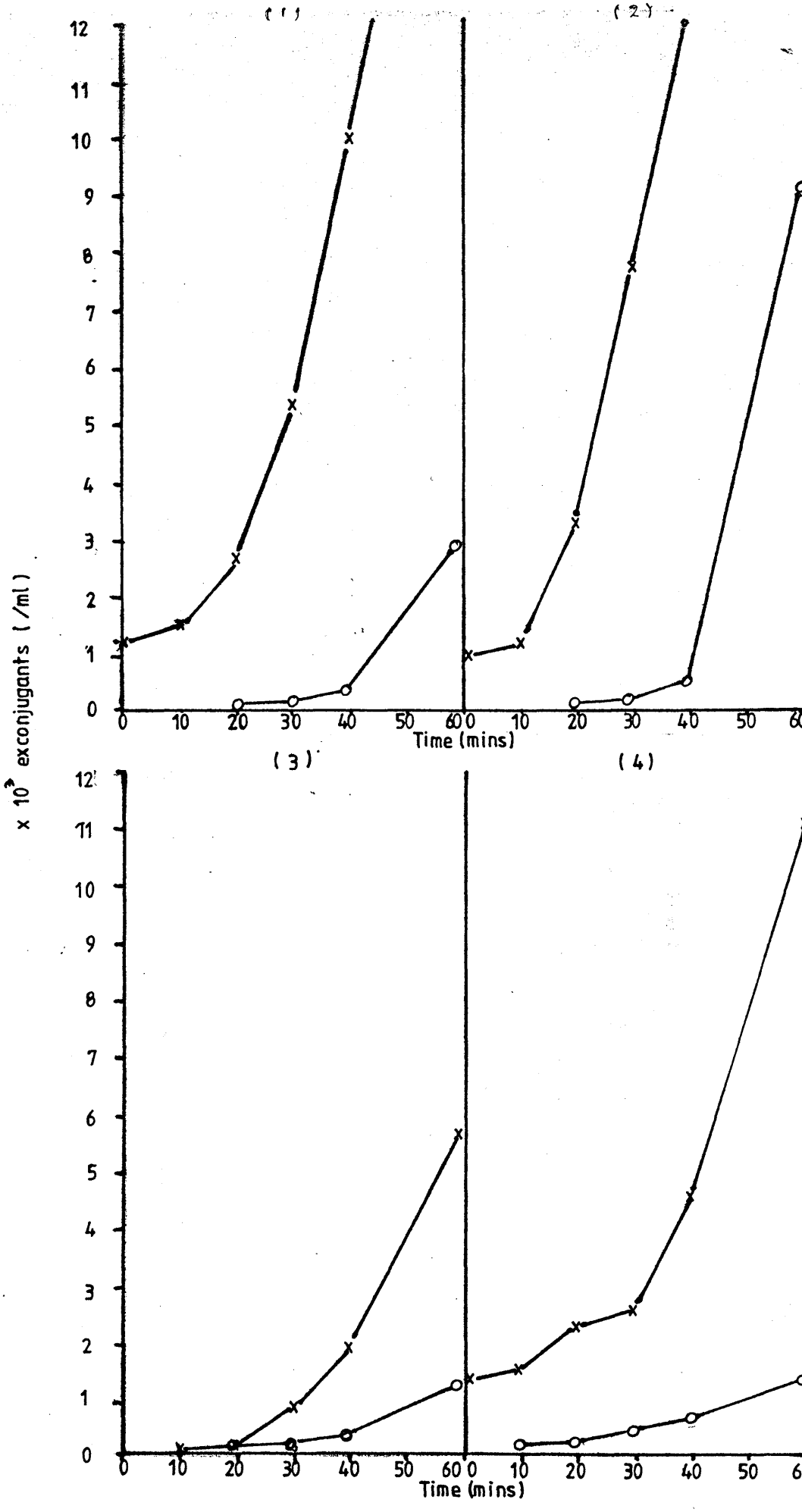
Time(mins)	tl^+	a^+
0	0	0
10	9×10^1	0
20	1.3×10^2	6×10^1
30	8.1×10^2	1.5×10^2
40	1.9×10^3	2.5×10^2
60	5.6×10^3	1.1×10^3

plotted on graph 3

Recombinant Class	No of recombinants DSX.1	DSX.2	DSX.330	DS.903
$\underline{arg}^+$ tl^+ xer^-	3	6	20	-
$\underline{arg}^+$ tl^+ xer^+	81	79	10	-
$\underline{arg}^+$ tl^- xer^-	4	4	14	-
$\underline{arg}^+$ tl^- xer^+	9	11	6	-
$\underline{arg}^+$ tl^+	-	-	-	44
$\underline{arg}^+$ tl^-	-	-	-	56

	DSX.1	DSX.2	DSX.330	DS.903
% recombination between arg & tl	13	15	40	56
% recombination between arg & xer	10	10	36	-

N.B. (i) recombinant that is underlined is the one selected for.
(ii) CGSC 5051 x DSX.1 and 2 has been repeated twice, whereas the crosses CGSC 5051 x DSX.330 and DS.903 have only been carried out once.



x - represents thr-leu^{+} exconjugants

o - represents arg^{+} exconjugants

CGSC 5051 x DSX.330 Nal^r + pCS202; is presented in Figure 6.5. From these crosses the time of entry for the genes xer1, xer2 and xerA mutant in DSX.1, DSX.2 and DSX.330 respectively, is approximately 85mins.

If the arg^+ excongugants were analysed for their Thr Leu phenotypes (Figure 6.5) a high linkage between arg and thr-leu was observed. This linkage is higher in DSX.1 and 2 than in DSX.330. To determine if this affect is real in DSX.330, this cross should be repeated, as it was for DSX.1 and 2. The genes argE and thr-leu are 10mins apart on the genetic map and therefore should behave in an unlinked fashion i.e. exhibit a % recombination of approximately 50% (Fincham, 1976). Why are argE and thr-leu showing such a high linkage? Is this a consequence of the Xer^- phenotype of DSX.1 and DSX.2? To determine whether Xer phenotype has any affect on the linkage between argE and thr-leu the identical cross was carried out with the xer^+ parent of the xer^- mutants, DS.903. The data for the cross CGSC 5051 x DS.903 is presented in Figure 6.5. The percentage recombination between argE and thr-leu for this cross was 56%, implying that these markers are unlinked as expected for argE and thr-leu. Why is there a difference in the apparent linkage between argE and thr-leu in the xer^- compared to the xer^+ recipient? There could be a number of explanations for this data, including: (i) that the Xer^- phenotype of DSX.1 and DSX.2 is affecting recombination between argE and thr-leu or (ii) that a chromosomal rearrangement has occurred between argE and thr-leu in the xer^- mutants compared to DS.903. This affect requires further investigation to determine what the cause of the high linkage between argE and thr-leu is in DSX.1 and DSX.2. To test if the Xer phenotype is causing this affect it would be necessary to clone the xer1/2 gene and use that clone to complement the Xer^- phenotype of DSX.1/2, to determine if the high linkage between argE and thr-leu still occurs.

When the cross data for DSX.1 and 2 was analysed, argE and xer show high linkage. However as already stated so to do argE and thr-leu. As arg and thr-leu show such high linkage one can not put much confidence in the data that argE and xer are highly linked, although if one examines the exconjugants which are arg^+ $thr\text{-}leu^-$, a higher proportion are xer^+ than xer^-. This implies that xer1/2 are more closely linked to arg than thr-leu. When the three point cross data for DSX.330 is examined 34% recombination between argE and xerA is observed. Other data (Section 6.4), indicates that xerA may lie after argE, hence in this cross argE may not be the distal marker. Therefore in DSX.330 the linkage between argE and xerA may be higher than that observed. The % recombination between argE and thr-leu is higher for DSX.330 than for DSX.1 and 2 but lower than that observed for DS.903. To determine if this is a real affect the cross CGSC 5051 x DSX.330 requires to be repeated. The Xer phenotype of exconjugants from the cross CGSC 5051 x DSX.330 should have correlated to Kan^r/Kan^s, but it did not. One possible explanation for this data is that Tn5 had transposed in the single colony of DSX.330 used in this mating.

6.4 Time of entry data for xerA.

In the xer^- mutant DSX.300, Tn5 and xer have been shown to be closely linked by P1 transduction. In these experiments 20 out of 20 Kan^r P1 transductants were also Xer^- in phenotype. Therefore the xerA gene mutant in DSX.300 can be mapped directly using the Tn5 marker Kan^r. The Hfr strain CGSC 4315 was P1 transduced for Kan^r using a P1 lysate made on DSX.300. The Kan^r transductants were found also to be Xer^- in phenotype. This xer^- Hfr strain was called DSX.380 and has its origin at 91mins transferring DNA in the clockwise direction (Figure 6.3). To verify that Tn5 is in xerA in this transductant, the wild type clone containing that

Figure 6.6 Data for the cross DSX.380 x DS.903.

DSX.380 x DS.903

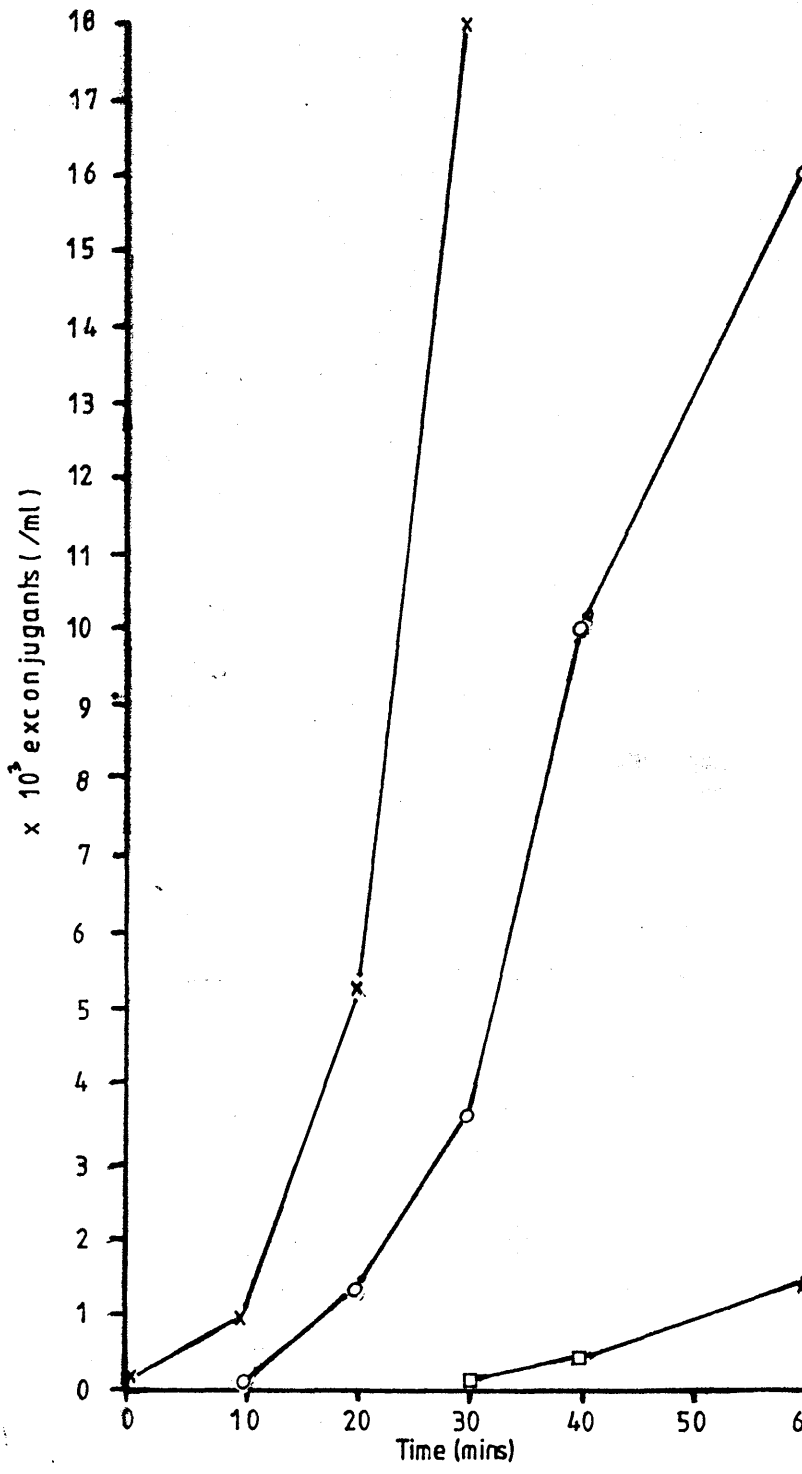
	No of exconjugants(/ml)				
Time(mins)	a^+	kan^r	xyl^+	a^+ Kan^r/tot	xyl^+ Kan^r/tot
0	$2.0x10^2$	0	0	0/20	-
10	$9.0x10^2$	$1.5x10^2$	0	1/8	-
20	$5.3x10^3$	$1.3x10^3$	0	0/42	-
30	$1.8x10^4$	$3.6x10^3$	$1.1x10^2$	2/48	4/11
40	$3.9x10^4$	$1.0x10^4$	$4.0x10^2$	3/47	23/29
60	$4.0x10^4$	$1.6x10^4$	$1.3x10^3$	5/45	25/50

Recombinant Class	No of Recombinants
$\underline{xyl}^+$ a^+ Kan^r	3
$\underline{xyl}^+$ a^- Kan^r	15
$\underline{xyl}^+$ a^+ Kan^s	18
$\underline{xyl}^+$ a^- Kan^s	24
$\underline{Kan}^r$ a^+ xyl^+	0
$\underline{Kan}^r$ a^+ xyl^-	0
$\underline{Kan}^r$ a^- xyl^+	0
$\underline{Kan}^r$ a^- xyl^-	50

%Recombination between xyl and arg = 58%
%Recombination between xyl and Kan = 84%

N.B. (i) recombinant underlined is the one selected for
(ii) six Kan^r exconjugants were tested for Xer phenotype and found to be Xer^-.
(iii) this cross has been repeated twice

(iv) in this experiment appropriate dilutions of the mating mixture were plated on L-agar and then replica plated onto L-agar + Kan. The time of entry of Kan was no different when time was allowed for expression.



x - represents arg^+ exconjugants

o - represents Kan^r exconjugants

□ - represents xyl^+ exconjugants

gene was used to carry out a *trans* complementation test. The wild type clone pGS30 complements the Xer$^-$ phenotype of DSX.380, confirming that this strain is *xerA*$^-$.

This Hfr strain was used to determine a time of entry map position for *xerA* by selecting for exconjugants which had received the kanamycin resistance gene. The recipient in this cross was DS.903 Nal^r Kan^s. The data for the interrupted mating of DSX.380 x DS.903 Nal^r is presented in Figure 6.6. These data imply that *xerA* which is closely linked to Tn5 has a time of entry map position of 85mins, just after *argE*. The genes *xyl* and *argE* exhibit a % recombination of 58%, which is consistent with them being unlinked. The time of entry data for Kan^r, positions *xerA* between *argE* and *xyl*, hence a proportion of the Xyl$^+$ exconjugants should be Kan^r. The marker *xyl* is distal to Kan^r in this cross, therefore one would expect either Xyl and Kan to be unlinked or to show a high linkage (low % recombination) as they are close. This is not the case, our data indicates a high % recombination, which could be explained by secondary transposition. If secondary transposition was occurring then the Kan^r exconjugants should be Xer$^+$ in phenotype. The Xer phenotype of six Kan^r exconjugants was tested using the pseudo-*cer* test plasmid, pCS202. These Kan^r exconjugants were found to be Xer$^+$ in phenotype, therefore secondary transposition could be occurring as Tn5 enters the recipient cell. Normally when a tranposon is present in a cell its tranposition is controlled as large numbers of transposons are disadvantageous. The transposon Tn5 regulates its transposition with a Tn5 encoded *trans* acting inhibitor (Berg and Berg, 1983). This inhibitor may act by complexing with the transposase protein or by binding to the recognition sites at the ends of Tn5, rendering them unavailable to transposase. In the Hfr strain DSX.380, Tn5 transposition would be inhibited, however when the chromosome is mobilized into a Tn5$^-$ recipient no inhibitor would be present hence

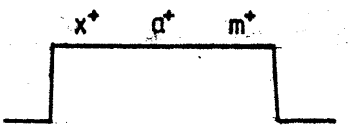
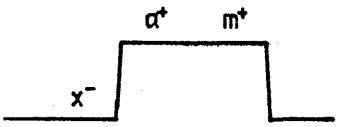
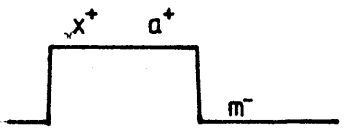
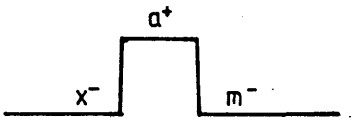
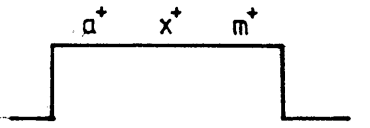
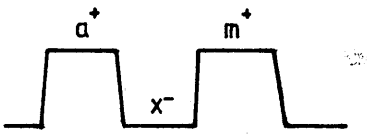
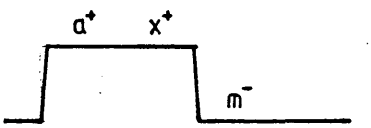
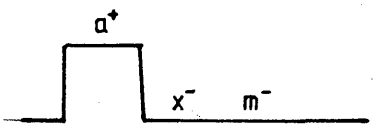
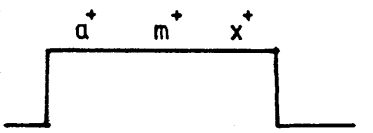
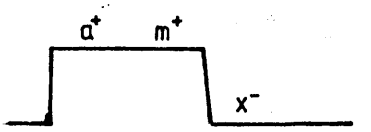
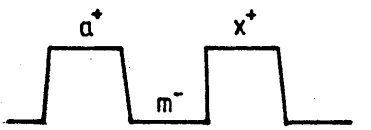
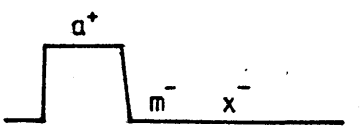
Figure 6.7 Three-point cross data for *xer*1

Recombinant class	(1)	(2)	(3)
arg$^+$ *mal*$^+$ *xer*$^+$	63	47	45
arg$^+$ *mal*$^+$ *xer*$^-$	24	21	21
arg$^+$ *mal*$^-$ *xer*$^+$	6	18	33
arg$^+$ *mal*$^-$ *xer*$^-$	7	10	4

(1), (2) and (3) represent three different experiments

By minimizing the number of crossovers, the order of genes was *xer*1, *arg*E and *mal*B. This was true for each of the three experiments analysed.

	(1)	(2)	(3)	$\bar{x}$	$\bar{x}$ ± S.E. (n)
%Recombination between *arg*E & *mal*B =	13	29	35	25·6	25·6 ± 3·8 (3)
%Recombination between *arg*E & *xer*1 =	31	32	24	29	29 ± 1·5 (3)

	Recombinant class	No of Recombinants (1)	(2)	(3)
Gene order (A) xer1 argE malB		63	47	45
		24	21	21
		6	18	33
		7	10	4
Gene order (B) argE xer1 malB		63	47	45
		24	21	21
		6	18	33
		7	10	4
Gene order (C) argE malB xer1		63	47	45
		24	21	21
		6	18	33
		7	10	4

⇒ Most probable gene order xer1 argE malB

transposition could occur. The genes argE and xerA should according to the time of entry of data for Kan^r show high linkage, as they are close to each other. When the Kan^r exconjugants were analysed for Arg (Figure 6.6), none of those tested were Arg^+. This may be due to the fact that when one selects for Kan^r exconjugants one could be selecting for a recombination event or a transposition event. If the frequency of Tn5 transposition is higher than the frequency of recombination of Tn5 into the recipients chromosome then exconjugants which have Tn5 recombined into the chromosome may be out numbered by those in which Tn5 has transposed.

6.5 Recombinational mapping for xer1

To detect the order of three genes the relative frequency of particular recombinational classes can be used to indicate linkage groups. If recombinational map units are related to time units approximately 10-15 recombination % units is equal to 1 time unit. Thus genes separated by a length of three or more minutes in conjugation time appear unlinked in recombinational analysis (Fincham, 1976). The markers used for the recombinational mapping of xer1 w ere argE, malB and xer1. Mapping studies have shown that DSX.1 is Mal^- and that this mutation maps to malB. The xer mutant, DSX.1 is $argE^-$, $malB^-$ and $xer1^-$, whereas the Hfr strain used, CGSC 4311 (Low,1973)(Figure 6.3) is $argE^+$, $malB^+$ and $xer1^+$. CGSC 4311 was mated with DSX.1 containing a pseudo-cer test plasmid for 80mins in a liquid mating. The exconjugants were selected for the transfer of argE and analysised for their Xer and Mal phenotypes. The results are presented in Figure 6.7. This data indicates that the gene order is xer1, argE and malB. The % recombination between argE and malB was $25.6\pm3.8\%(3)$, whereas that between argE and xer1 was $29\pm1.5\%(3)$. The actual number of map units between argE and malB

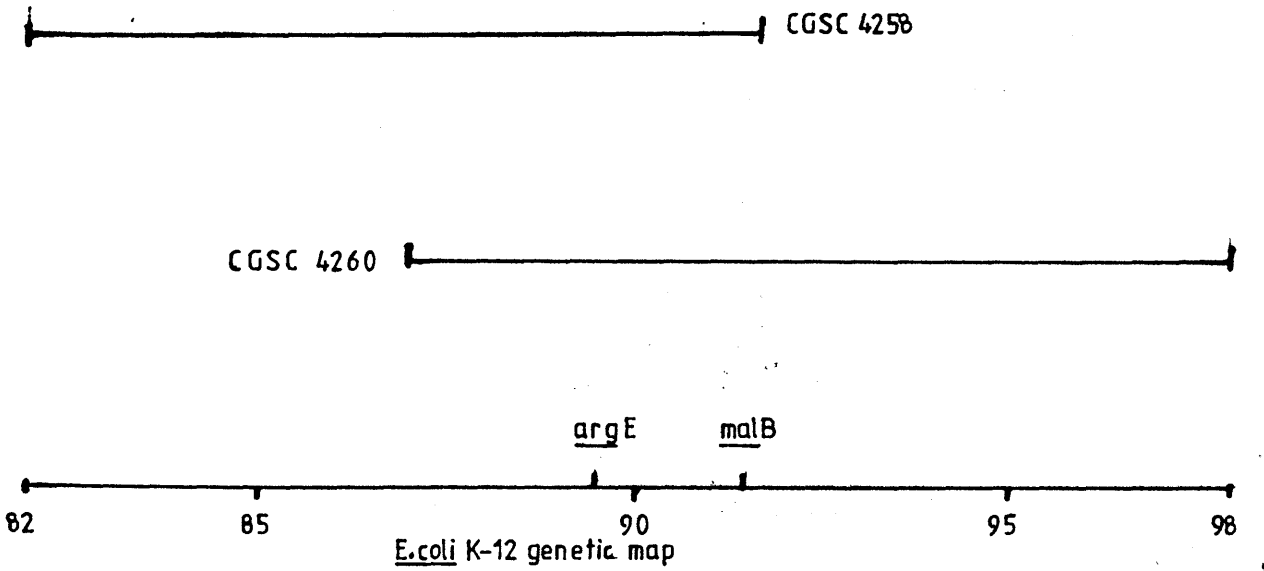


Figure 6.8 Illustration of the regions of the E.coli K-12 chromosome carried by the F's, CGSC 4258 and CGSC 4260.

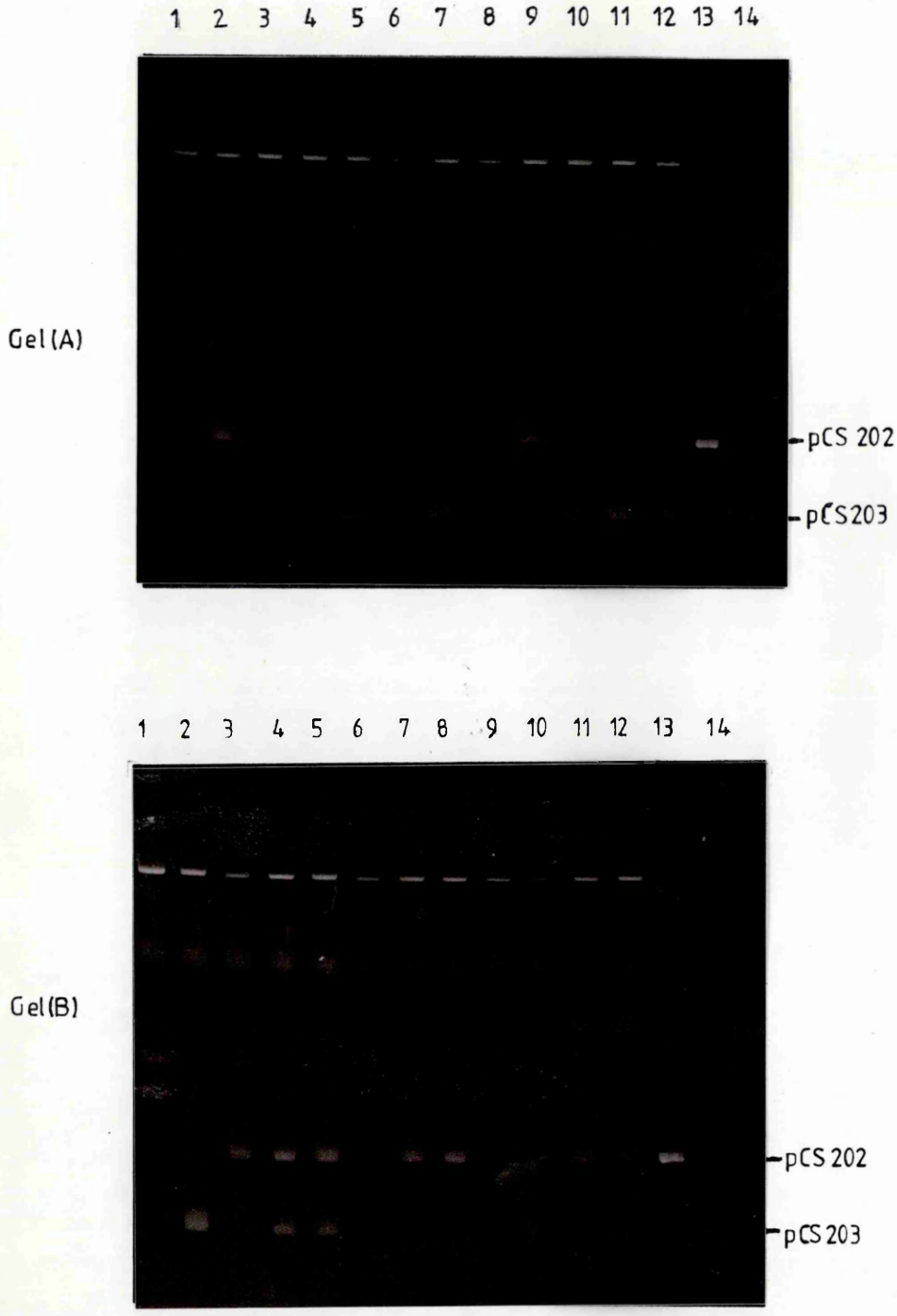


Figure 6.9 Analysis of the Xer phenotype of exconjugants from the crosses F'4258 x DSX.1 and F'4260 x DSX.1

Gel(A) F'4258 x DSX.1 + pCS202

Lanes (1-12) exconjugants from the cross F'4258 x DSX.1

(13) pCS202

(14) pCS203

Gel(B) F'4260 x DSX.1 + pCS202

Lanes (1-12) exconjugants from the cross F'4260 x DSX.1

(13) pCS202

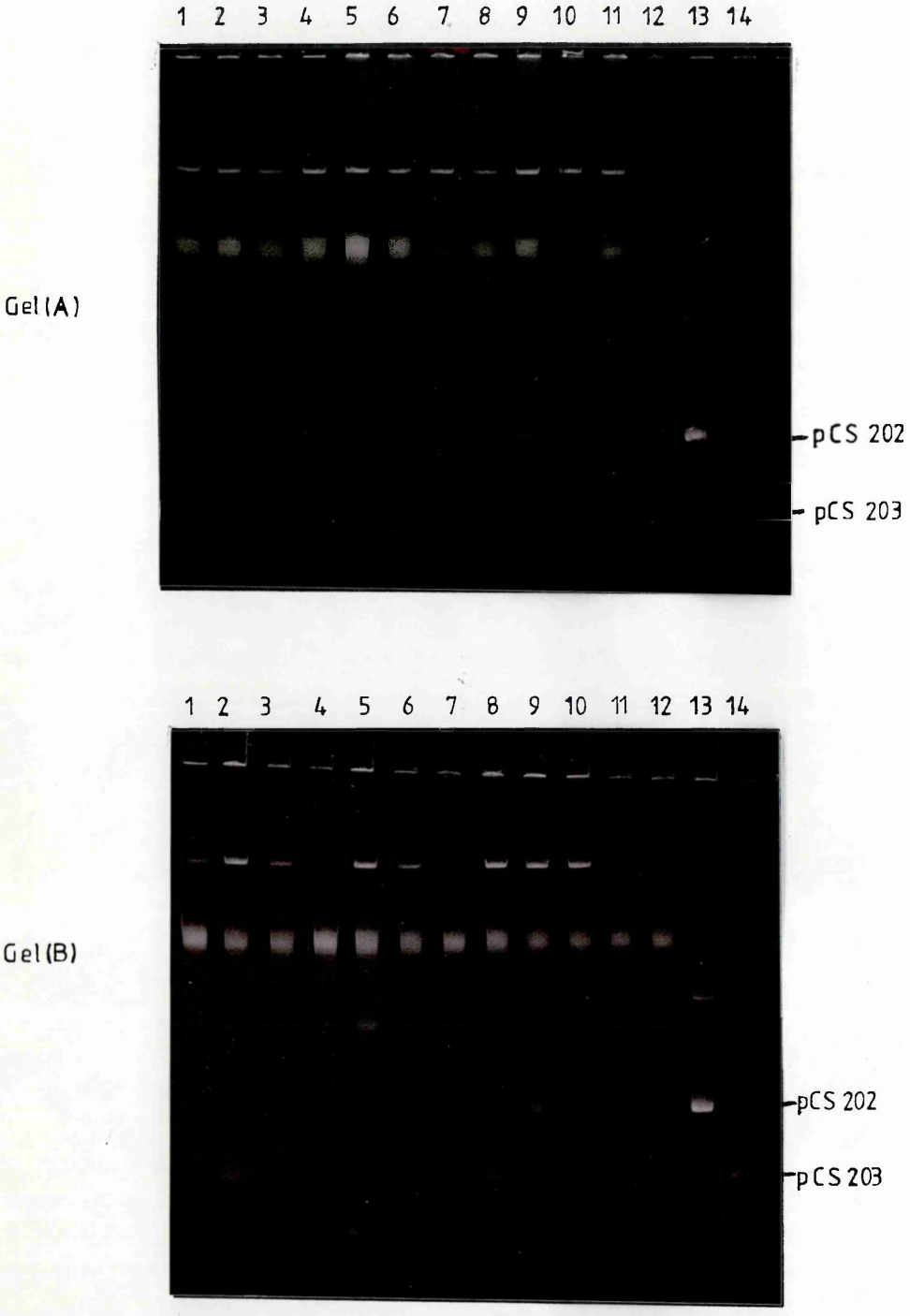


Figure 6.10 Analysis if the Xer phenotype of exconjugants from the crosses, F'4258 x DSX.2 and F'4260 x DSX.2.

Gel(A) F'4258 x DSX.2 + pCS202

Lanes (1-12) exconjugants from the cross F'4258 x DSX.2

(13) pCS202

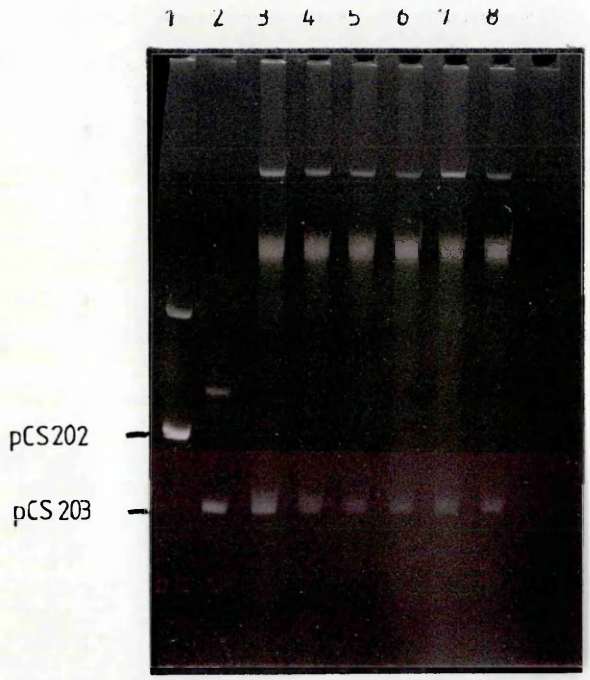
(14) pCS203

Gel(B) F'4260 x DSX.2 + pCS202

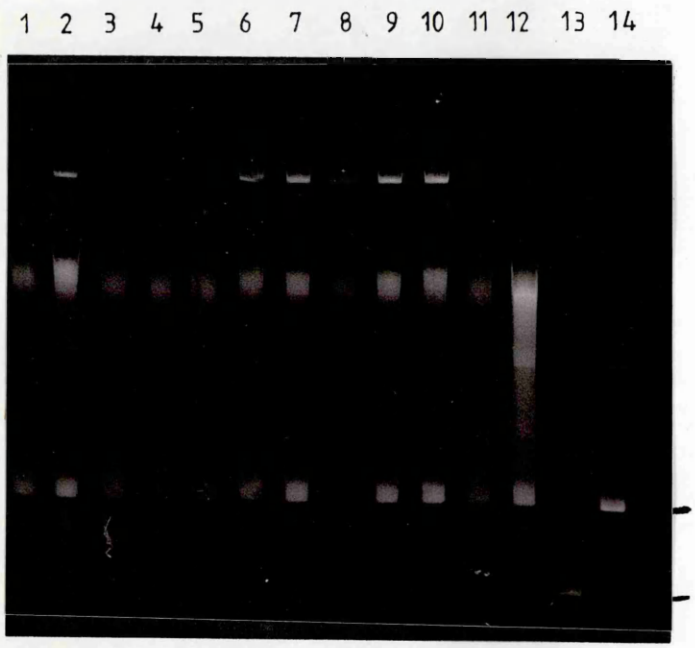
Lanes (1-12) exconjugants from the cross F'4260 x DSX.2

(13) pCS202

(14) pCS203



Gel (A)



Gel (B)

Figure 6.11 Analysis of the Xer phenotype of exconjugants from the crosses, F'4258 x DSX.330 and F'4260 x DSX.330.

Gel(A) F'4258 x DSX.330 + pCS202

Lanes (3-8) exconjugants from the cross F'4258 x DSX.330

(2) pCS203

(1) pCS202

Gel(B) F'4260 x DSX.330 + pCS202

Lanes (1-12) exconjugants from the cross F'4260 x DSX.330

(13) pCS203

(14) pCS202

is 2, therefore xer1 probably lies 2 map units to the other side of argE. However in this cross the marker selected for was argE, which is not the most distal marker, therefore one can not say that all exconjugants have received xer1, hence xer1 may actually be closer to argE than the data suggests.

6.6 F prime complementation data.

The time of entry data for the xer genes mutant in DSX.1, DSX.2 and DSX.330 would place these genes at 85mins^{+} 5mins. Available to us were two F' covering the area from 82 to 98mins, called CGSC 4258 (Low 1972) and CGSC 4260 (Low 1972) (Figure 6.8). The F' 4258 is reported to carry the region from 82 to 92mins, whereas F'4260 carries the region from 87 to 98mins. The regions that these F' were reported to carry was verified using the markers recF, argE and malB. This confirmed that the F'4258 carries from 83 to 91.5mins and that F' 4260 carries at least from 89 to 91mins. Both these F' carry argE, therefore one can select for the transfer of these F's using argE, as the recipient xer^{-} strains are auxotrophic for this marker.

The Xer phenotype of the Arg^{+} exconjugants from the following crosses were analysed, using single colony gels:-

(1) F'4258 x DSX.1 Nal^{r} + pCS202 (Figure 6.9A)

(2) F'4260 x DSX.1 Nal^{r} + pCS202 (Figure 6.9B)

(3) F'4258 x DSX.2 Nal^{r} + pCS202 (Figure 6.10A)

(4) F'4260 x DSX.2 Nal^{r} + pCS202 (Figure 6.10B)

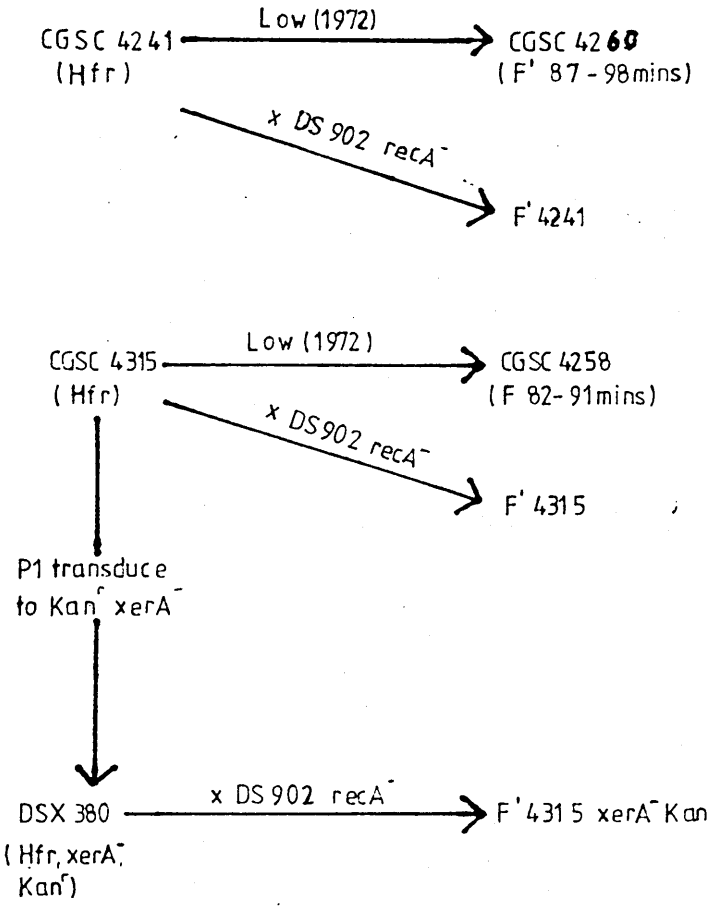
(5) F'4258 x DSX.330 Nal^{r} + pCS202 (Figure 6.11A)

(6) F'4260 x DSX.330 Nal^{r} + pCS202 (Figure 6.11B)

The F'4258 complements the Xer^{-} phenotype of DSX.330, whereas F'4260 does not. This implies that the xerA gene mutant in DSX.330 lies between 82 and 87mins, which is consistent with the time of entry data for xerA.

Both the F primes tested complement the Xer^{-} phenotype of DSX.1

and 2, although to varying degrees, implying that xer1 and 2 lie between 87 and 91mins, which is consistent with the time of entry data. The F'4258 complements the Xer$^-$ phenotype of DSX.1 and 2 in a larger proportion of the exconjugants examined than does F'4260. The three point cross data for the xer1 gene mutant in DSX.1 places that gene at approximately 87mins. The F'4258 carries the region from 82 to 92mins and therefore has the sequences upstream and downstream of the xer1 gene. On the other hand F'4260 carries the region from 87 to 98mins and therefore does not carry the sequences to one side of the xer1 gene. Hence regulation of transcription/translation of the wild type xer1 gene may be altered in the F'4260 compared to F'4258, which could account for the differences in complementation observed. The recipient strains in these crosses are recA$^+$, recF$^-$, therefore the potential for gene conversion ex ists, which may also be affecting this data. The pseudo-cer dimer in the Arg$^+$ exconjugants from the crosses F'4258 and F'4260 x DSX.1 and 2 was present in different exconjugants in varying proportions of parental to deletion forms. In the cross F'4258 x DSX.330 the pseudo-cer dimer was all broken down to the deletion form. Why the difference in the behaviour of the test plasmid in these trans complementation tests? The xer$^-$ mutant DSX.330 contains a Tn5 insertion in the xerA gene whereas DSX.2 may be a spontaneous mutant. The exact nature of the xer mutation in DSX.1 is unknown, therefore there is a possibility that DSX.1 is also a spontaneous mutant. If one speculates that the xer mutations in DSX.1 and 2 are spontaneous then they could be deletion or point mutations. This implies that in DSX.1 and 2 a mutant xer gene product could be produced and a wild type xer gene product from the F'. If these two gene products both bind to cer sites then there will be competition for binding, but only the wild type gene product will promote cer-specific recombination. Another possible explanation could be that the



	COMPLEMENTATION		
	DSX 1	DSX 2	DSX 330
CGSC 4241 (Hfr)	+/− (12)	+/− (12)	− (12)
F'4241	+/− (24)	+/− (24)	NT
CGSC 4315 (Hfr)	++/− (12)	++/− (12)	++ (12)
F'4315	++/− (24)	++/− (24)	++/− (12)
DSX 380 (Hfr, xerA⁻, Kan^r)	++/− (12)	++/− (12)	− (12)

Figure 6.12 Summary of the F' complementation data for the *xer*⁻ mutants DSX.1, DSX.2 and DSX.330.

++ = complementation in all exconjugants examined

++/- = complementation in > 50% of exconjugants examined

+/- = complementation in < 50% of exconjugants examined

- = no complementation in any exconjugant examined

Figures in ( ) represent the number of exconjugants examined

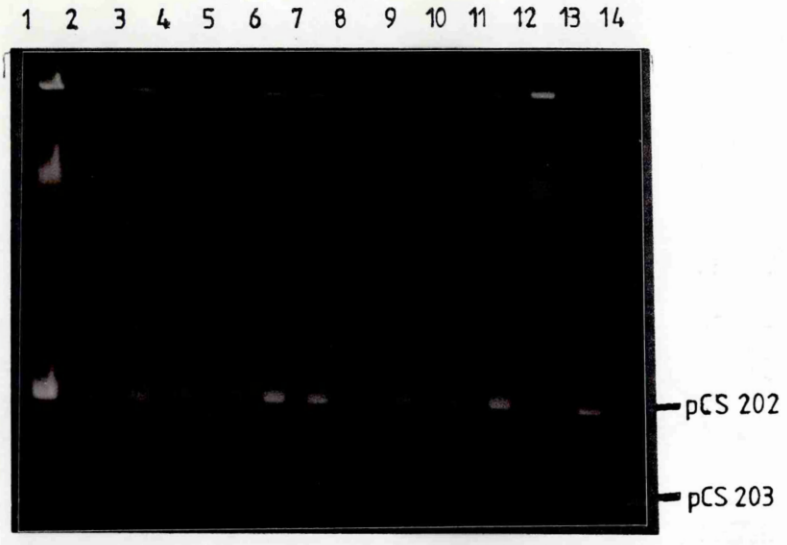


Figure 6.13 Analysis of the Xer phenotype of exconjugants from the cross F'5505 x DSX.1, DSX.2 and DSX.330.

Lanes (1-4) exconjugants from the cross F'5505 x DSX.1

(5-8) exconjugants from the cross F'5505 x DSX.2

(9-12) exconjugants from the cross F'5505 x DSX.330

(13) pCS202

(14) pCS203

xer1/2 gene(s) is autoregulated, so that the presence of a mutant gene product would repress synthesis. Alternatively the xer1/2 gene product could be functioning as a dimer. If hetrodimers of mutant and wild type xer1/2 gene product were formed, they may mediate cer-specific recombination less efficiently than a wild type dimer. This could lead to the mixed phenotype of the two cer test plasmid observed in the trans complementation tests using the F'4258 and F'4260. Alternatively there could be other possible explanations depending upon the properties of the xer genes.

F's from the same region of the chromosome as F'4258 and F'4260 were constructed by crossing the Hfr strains CGSC 4315, CGSC 4241 and DSX.380 with DS. 902 (recA$^-$) to form F'4315, F'4241 and F'4315(xerA$^-$). A summary of the F' complementation of the Xer$^-$ phenotypes of DSX.1, DSX.2 and DSX.330 is presented in Figure 6.12. The analogous F's 4315 (this work) and 4258 (Low, 1973) complemented in a higher number of exconjugants examined than the analogous F's 4241 (this work) and 4260 (Low, 1973) do. The F' 4315 (xerA$^-$) (this work), complements the Xer$^-$ phenotype of DSX.1 and 2 but not that of DSX.330, as expected. This confirms that at least two host complementation groups are required for cer-specific recombination, as predicted from the complementation data with the wild type xerA plasmid clone (Chapter 5).

To test the hypothesis that F' transfer itself produces a Xer$^+$ phenotype an F' carrying a different region of the chromosome was mobilized into DSX.1, DSX.2 and DSX.330. The F' used, CGSC 5505 (Novel and Novel, 1976) is reported to carry the region from 34 to 45mins. This F' carries his and DSX.1, DSX.2 and DSX.330 are his$^-$, therefore transfer of the F' can be selected for using his. The Xer phenotye of these exconjugants was examined on single colony gels (Figure 6.13). These gels show that transfer of this F' does not affect the Xer phenotype of DSX.1, DSX.2 and DSX.330 i.e. pCS202 was in the addition form in these exconjugants.

Therefore one can say that F' transfer itself does not affect the Xer$^-$ phenotype of the recipients.

These F' *trans* complementation tests are not ideal as the recipients in these crosses are *rec*A$^+$. The presence of the *rec*A gene product could be causing gene conversion, hence one can not conclusively state that *trans* complementation is occurring, however these data give an indication of the map positions of the *xer* genes. If more time had been available *rec*A$^-$ derivatives of the *xer* mutants would have been constructed. These data imply that the *xer*A gene mutant in DSX.300 lies between 82 and 87mins and that the *xer*1/2 gene(s) mutant in DSX.1 and 2 lie between 87 and 91 mins.

Discussion and Conclusions.

The time of entry data places the *xer* genes mutant in DSX.1, DSX.2 and DSX.330 at approximately 85mins. In the interrupted matings of DSX.1 and 2 with CGSC5051 a high proportion of the *arg*$^+$ exconjugants were Xer$^+$ in phenotype. It is possible that the *xer*$^+$ exconjugants are more viable than the *xer*$^-$ ones. This could be explained if the *xer*1/2 gene product(s) has more than one phenotype and is in some way involved in viability after conjugation. To test if the Xer$^-$ phenotype of DSX.1/2 is involved in such an affect it would be necessary to clone the wild type *xer*1/2 gene(s) and use that clone to complement the Xer$^-$ phenotype.

An attempt was made to use recombinational mapping to determine the linkage of *xer*1, however only one marker was selected, *arg*. This is not ideal as the *arg* marker was used in the three point recombination analysis. To improve this analysis a distal marker, such as *xyl* should have been selected and the exconjugants analysed for their Xer, Arg and Mal phenotypes.

F' complementation data was used to obtain confirmation of these map positions. The F's CGSC 4258 and CGSC 4260 overlap in the region of 87-91mins and both complement to varying degrees the Xer$^-$ phenotype of DSX.1 and 2, implying that the *xer*1/2 gene lies between 87-91mins. F'4260 complements the Xer$^-$ phenotype of DSX.1 and 2 in a smaller number of exconjugants examined than does F'4258. This can be explained by postulating that the *xer* gene mutant in DSX.1/2 lies at the 87min boundary of the F'4260, as indicated by the three point cross data for *xer*1. If *xer*1 lies at the boundary of the F'4260 then the *xer*1 gene may be being transcribed and/or translated less efficiently, hence *cer*-specific recombination may be less efficient. In the crosses with the F'4258 and F'4260 x DSX.1 and 2 the pseudo-*cer* test plasmid is present in different exconjugants in different proportions of addition and deletion forms. This supports the idea that in these exconjugants a mutant and wild type *xer* gene product are present and could either be competing for *cer* sites, autoregulating their own expression or forming less functional hetro-dimers. The behaviour of the two *cer* test plasmids in these F' complementation tests is reminiscent of their behaviour in W5445 (Chapter 3).

The heterogeneous phenotype of the pseudo-*cer* dimers within exconjugants could be explained by the mixing of the plus and minus subunits of the *xer* encoded protein. The differences in the behaviour of the pseudo-*cer* dimers between different exconjugants i.e. a proportion of the *arg*$^+$ exconjugants are *xer*$^+$ and the rest are *xer*$^-$, could be explained by gene conversion. This possibility could be tested by curing the exconjugant of the F' and then determining its Xer phenotype. Alternatively if *recA*$^-$ *xer*$^-$ strains were used as recipients gene conversion could not occur.

The F'4258 complements the Xer$^-$ phenotype of DSX.330 in every exconjugant examined, whereas the F'4260 does not. This places

the xerA gene, mutant in DSX.330 between 82 and 87mins. The pseudo-cer test plasmid from the cross F'4258 x DSX.330 is all present in the deletion form, which contrasts to the situation in DSX.1/2. The mutant DSX.330 contains Tn5 within the xerA gene, hence no mutant gene product will be produced, whereas in DSX.1/2 a mutant gene product may be produced.The difference in the Xer complementation observed between DSX.1/2 and DSX.330 could be due to the nature of the mutations in these strains.

The xerA3 gene, was found to be recessive to the wild type gene. The question of dominance remains unanswered for xer1 and 2. The simplest way to test the dominance of the xer1 and 2 mutant alleles is to carry out a complementation test using a cloned wild type allele of the gene(s). If the xer1 allele is dominant, then when complementation with the xer1$^+$ clone occurs the resulting phenotype would be Xer$^-$, however if the xer1 mutation is recessive the resulting phenotype would be Xer$^+$.

In Chapter 5 it was observed that the wild type xerA$^+$ plasmid clone complements the Xer$^-$ phenotype of DSX.330 but not that of DSX.1/2, implying that two host complementation groups are required for cer-specific recombination. This was confirmed using the F'4315(xerA$^-$), which complements the Xer$^-$ phenotype of DSX.1 and 2 but not that of DSX.330.

The F' complementation tests are not conclusive as the recipients are recA$^+$. Therefore one can not conclusively state that trans complementation is occurring, however these data give some indication of the map position of the xer genes. If more time had been available recA$^-$ derivatives of these xer$^-$ mutants would have been constructed. To map the position of the xer genes more precisely P1 transduction could be used.

Colin Stirling is in the process of deriving more Tn5::xer insertion mutants using λ 467 (Kleckner, 1977). A total of ten Tn5 insertion mutants have so far been derived, two of which are complemented by the plasmid clone containing the xerA gene. It

is hoped that these new xer mutants will be Tn5 insertion mutants in xer genes, which simplifies cloning and mapping of the genes. Ideally one would like to obtain Tn5 insertions in the xer genes mutant in DSX.1 and DSX.2.

Now that an approximate map position for the xer genes has been determined, one can compare these host factors with host factors required for other site-specific recombination systems. This will allow us to say whether the xer gene products are used in any other characterised systems.

System	Factor	Protein size (kd)	Map Position (mins)
λ int	IHF himA	11.2	37
	himD	9.5	20
Mu gin	FIS	N.K.	N.K.
Salm hin	Factor II	12	N.K.
	HU	9.9	N.K.
ColE1	xerA	17	82-87
cer	xer1/2	N.K.	87-91

N.K. = not known

This table is not complete but from it one can say the following:-

(i) xerA is not himA, himD, Factor II or HU.

(ii) xer1/2 is not himA or himD.

(iii) cannot say anything about the xer genes and FIS as the protein size and map position are unknown.

(iv) cannot say anything about xer1/2 with respect to Factor II, or HU as the size of the protein product of xer1/2 is as yet unknown.

The region of the E.coli K-12 genetic map to which these genes map has been well characterized. The xerA gene sequence has been

checked against sequenced E.coli genes in the EMBO gene bank and no significant homology has been detected. This region of the chromosome contains genes involved in:

(i) homologous recombination- recF (83mins), recG (82mins), recP (85mins) and recQ (85mins)

(ii) DNA replication and synthesis- oriC (84mins), dnaA & N (83mins), gyrB (83mins), polA (86.5mins) and ssb (92mins)

(iii) coding for the large and small ribosomal subunits

(iv) other miscellaneous genes, including nutritional genes e.g. malB (91.5mins), argE (89mins).

Hill et al (1977) has also reported that this region of the chromosome undergoes chromosomal rearrangements resulting from recombination between ribosomal RNA genes.

CHAPTER 7

CONCLUDING REMARKS

The aim of this project was to determine the origin of the cer-specific recombinase. Site-specific recombinase genes are usually positioned adjacent to the site at which they act (Nash and Pollock, 1983), hence the most likely ORFs are the cer-adjacent ones. However preliminary experiments suggested that the recombinase is not encoded by ColE1 but is chromosomally encoded. Therefore Tn5 mutagenesis was used to obtain host mutants, which are deficient in cer-specific recombination. A total of ten Tn5::xer mutants have been isolated, three of which have been studied in detail. These three xer mutants are DSX.1, DSX.2 and DSX.300.

Summers and Sherratt (1984) demonstrated a correlation between multimerization and instability for pACYC184. The xer mutants were used to confirm that relationship for ColE1 itself. Indicating that the cer-specific multimer resolution system is a major stability determinant of ColE1. xer mutants are also defective for the multimer resolution systems of other high copy number plasmids, including ColK, CloDF13 and perhaps ColE2 and ColE3. Implying that a common mechanism of multimer resolution may be used by related high copy plasmids to enhance their stability.

A wild type plasmid clone containing an 8.5kb EcoRI-HindIII fragment was isolated, which when present in trans, complements the Xer^- phenotype of DSX.300. This complementing function was further localized to a 0.95kb AccI-SphI fragment, which complements a $recA^-$ derivative of DSX.300. This minimum complementing clone pGS38, was sequenced by Colin Stirling. Sequence analysis revealed an ORF encoding for a polypeptide of 17kd, which has a good consensus ribosome binding site, two alternative promoters and a terminator. A polypeptide was visualized on whole cell Laemmli protein gels and was confirmed as an insert specific polypeptide using minicells.

The largest plasmid clone containing the xerA gene does not

complement the Xer⁻ phenotype of DSX.1 and DSX.2, defining at least two host complementation groups necessary for cer-specific recombination. An F prime which carries a mutant xerA gene complements the Xer⁻ phenotype of DSX.1 and 2 but not that of DSX.300, confirming that at least two host complementation groups are required.

Classical genetic techniques were used to determine the genetic map position of the xer genes. The data indicates that xerA, which is mutant in DSX.300 lies between 82 and 87mins, whereas the xer1 and 2 genes mutant in DSX.1 and 2 lie between 87 and 91mins. If more time had been available one could have further defined the position of the xer genes using P1 transduction.

A requirement for host factors has been observed in other site-specific recombination systems, such as the lambda integration system and some of the inversion systems, including the type I fimbriation system. The role of the host factors in cer-specific recombination is as yet unknown, but one could postulate that one gene product is the recombinase and that the other may be an acessory factor. IHF is postulated by Lange-Gustafsson (1984) to enhance the activity of Int, indicating that IHF acts as an acessory factor in lambda integration. One of the Xer gene products may like IHF, act as an acessory factor by producing bends in the DNA (Wu and Crothers, 1984) or by assisting in the formation of higher order structures by protein-DNA interactions. The cross-over region of the cer-site specific recombination was identified as a 35bp region from 3906-3946bp (Summers et al, 1985) and yet the minimum functional cer fragment is 240bp in length, implying that sequences in addition to the crossover region are required. The functional cer fragment may be larger than the defined crossover region as it contains binding sites for acessory factors as seen in the attP site involved in lambda integration, which has three IHF binding sites interspersed with four Int binding sites (Craig and Nash, 1984).

Site-specific recombination systems such as lambda integration and the inversion systems utilize host factors to enhance site-specific recombination. The host factor IHF was initially shown to be involved in lambda integration but subsequent work has indicated that it is involved in lambda encapsidation and replication of pSC101 (Gamas et al, 1986). Perhaps host factors involved in site-specific recombination reactions may be used in more than one system, hence the xer genes were compared to host factors used in other site-specific systems. The genetic map position and the size of the polypeptide encoded by the xerA gene indicate that this gene is not himA, himD, FactorII or HU. The map position of the xer1 and 2 genes indicates that they are not himA or himD (Kikuchi et al, 1985; Miller and Nash, 1981).

To determine if the xer genes are utilized by any other site-specific recombination system it would be necessary to test each system in the xer mutants. However data for IHF (Lange-Gustafsson and Nash, 1984) indicates that it acts to enhance integration and is not essential, hence the affect of xer functions on other site-specific recombination systems may have to be tested in vitro.

Site-specific recombination systems are normally characterized by developing in vitro systems e.g. the lambda integration system (Nash, 1975). This approach could be useful to characterize the components of cer-specific recombination system and to determine the roles of the host functions. Nuclease protection studies could also be used to determine the binding sites of the recombinase and acessory factors. In addition nucleases protection studies would define the site of strand exchange in the cer-specific recombination system.

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Gel (A)

Lanes (1-3) ColE1 + pCS202 in DSX.1

(4) pCS202

(5) pCS203

(6) ColE1

(7-9) ColE1 + pCS202 in DSX.2

Gel (B)

Lanes (1-4) ColE1 + pCS202 in DSX.330

(5) pCS202 in DSX.330

(6) pCS202

(7) pCS203

(8) ColE1