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Xer-mediated site-specific recombination at *cer* generates Holliday junctions *in vivo*

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Normal segregation of the *Escherichia coli* chromosome and stable inheritance of multicopy plasmids such as ColE1 requires the Xer site-specific recombination system. Two putative λ integrase family recombinases, XerC and XerD, participate in the recombination reactions. We have constructed an *E.coli* strain in which the expression of *xerC* can be tightly regulated, thereby allowing the analysis of controlled recombination reactions *in vivo*. Xer-mediated recombination in this strain generates Holliday junction-containing DNA molecules in which a specific pair of strands has been exchanged in addition to complete recombinant products. This suggests that Xer site-specific recombination utilizes a strand exchange mechanism similar or identical to that of other members of the λ integrase family of recombination systems. The controlled *in vivo* recombination reaction at *cer* requires recombinase and two accessory proteins, ArgR and PepA. Generation of Holliday junctions and recombinant products is equally efficient in RuvC$^-$ and RuvC$^+$ cells, and in cells containing a multicopy RuvC$^+$ plasmid. Controlled XerC expression is also used to analyse the efficiency of recombination between variant *cer* sites containing sequence alterations and heterologies within their central regions.

Key words: Holliday junction/site-specific recombination/ Xer

Introduction

Xer site-specific recombination is required to ensure both the stable inheritance of multicopy plasmids and efficient segregation of the *Escherichia coli* chromosome at cell division (Summers and Sherratt, 1984; Blakely *et al.*, 1991). Instability of naturally occurring plasmids can arise as a consequence of the generation of plasmid multimers through homologous recombination (Summers and Sherratt, 1984). To counteract this, ColE1 and many other plasmids contain a locus of ~220 bp (for example, *cer* in ColE1) at which host-encoded proteins act to monomerize the multimers by site-specific recombination (Summers *et al.*, 1985; Stirling *et al.*, 1988a,b, 1989; Colloms *et al.*, 1990; Blakely *et al.*, 1993).

Homologous recombination between replicating or newly replicated *E.coli* chromosomes can create chromosomal dimers, the monomer components of which will be unable to be segregated into daughter cells as the host cell divides. A locus, *dif*, present in the terminus region of the *E.coli* chromosome, and with sequence homology to *cer*, is a substrate for Xer-catalysed site-specific recombination (Blakely *et al.*, 1991; Kuempel *et al.*, 1991). These workers have proposed that Xer-mediated recombination at *dif* converts chromosomal dimers to monomers and thereby allows their segregation at cell division.

The proteins which mediate Xer site-specific recombination are encoded by the *E.coli* chromosome. At the outset of the work described here, three proteins were known to be required for Xer-mediated recombination at *cer*, while only one of these, the putative recombinase XerC, appeared necessary for recombination at *dif*. XerC binds to *cer* and *dif* sites *in vitro* and has sequence homologies with the λ integrase family of site-specific recombinases (Colloms *et al.*, 1990; Blakely *et al.*, 1991, 1993). Genetic evidence suggests that the other two proteins have accessory but necessary roles in ensuring that recombination at *cer* only occurs intramolecularly (Summers, 1989). One of these accessory proteins, ArgR, binds to *cer* ~100 bp upstream of the position of strand exchange (Stirling *et al.*, 1988b) and additionally controls the expression of the arginine biosynthetic genes (Maas, 1961; Glansdorff, 1987). The other accessory protein, PepA, is an amino-exopeptidase whose detailed mechanistic role in Xer recombination remains unclear (Stirling *et al.*, 1989). We have recently shown that the product of a further gene, *xerD*, is also required for recombination at *cer* and *dif* (Blakely *et al.*, 1993). XerD protein shows 37% amino acid sequence identity to XerC and, like XerC, appears to be involved in catalysis of strand exchange (Lovett and Kolodner, 1991; Blakely *et al.*, 1993).

The different protein requirements of Xer-mediated recombination at *cer* and *dif* are mirrored by the organization of the two sites and by differences in their recombination characteristics. A functional *dif* site, sufficient for both XerC and XerD binding and able to support the Xer recombination reaction, consists of only 30 bp (Blakely *et al.*, 1991, 1993). Approximately 30 bp of *cer* with substantial sequence homology to *dif* constitutes the 'core' of the *cer* recombination site to which XerC and XerD bind and within which recombination occurs, while the remaining 190 bp are 'accessory sequences' (Summers, 1989; Blakely *et al.*, 1993). Because *dif* sites are able to recombine both inter- and intra-molecularly whilst *cer* sites will only recombine when present on the same DNA molecule, the accessory sequences, along with the accessory proteins, are implicated in ensuring that Xer recombination at *cer* is intramolecular.

Here we report the construction of an *E.coli* strain in which we are able to regulate tightly the expression of *xerC*, therefore allowing us to perform controlled Xer recombination reactions *in vivo*. Analysis of the products

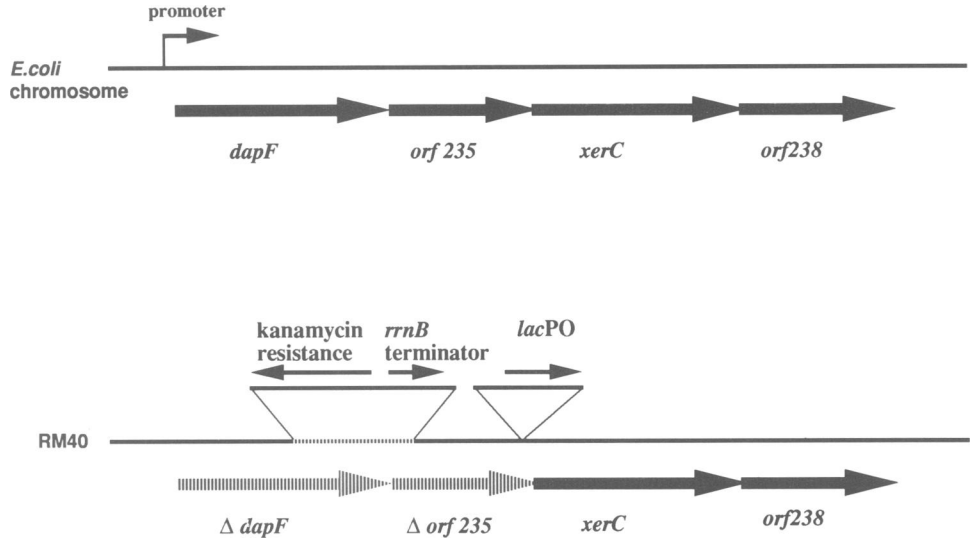


Fig. 1. Comparison of the *xerC* locus in wild type *E.coli* and in the strain RM40. The chromosomal organization of the four genes comprising the *xerC*-containing transcriptional unit in wild type *E.coli* and in strain RM40 is depicted (arrows denote the direction of transcription). Transcription of *xerC* and *orf238* in RM40 is derived from the *lac* promoter, which has been inserted within the coding sequence of *orf235*. The genes encoding DapF and ORF235 have been partially deleted (indicated by Δ) by insertion of the kanamycin resistance gene of Tn*903* and the *rrnB* transcriptional terminator, thus allowing selection for RM40 and enhancing the transcriptional regulation of *xerC* respectively.

of Xer-mediated recombination at *cer* in this strain has demonstrated the production of Holliday junctions which have been created by the exchange of a specific pair of DNA strands within the substrate plasmid. These results indicate that Xer site-specific recombination has a reaction mechanism similar or identical to that of other λ integrase family systems [see Landy (1989) and Stark *et al.* (1992) for reviews].

Results

Controlled Xer site-specific recombination in vivo

In order to study the Xer recombination reaction *in vivo*, an *E.coli* K-12 strain, RM40, in which the expression of *xerC* can be tightly regulated, was constructed (see Figure 1). In its natural chromosomal position, *xerC* is believed to be co-transcribed with the genes *dapF*, *orf235* and *orf238* (Richaud *et al.*, 1987; Colloms *et al.*, 1990). The promoter directing transcription of these genes has been mapped to ~280 bp upstream of *dapF* (Richaud and Printz, 1988). In RM40, transcription of *xerC* is initiated from the *lac* promoter and controlled by the *lac* operator, both of which have been inserted into the open reading frame of *orf235*. In addition, parts of the genes encoding DapF and ORF235 were substituted by the Tn*903*-derived kanamycin resistance gene and the *rrnB* transcriptional terminator (Figure 1). These insertions create a selection for the strain and reduce transcriptional readthrough from the natural *dapF* promoter respectively. The functions of *dapF*, *orf235* and *orf238* are not essential for Xer site-specific recombination (our unpublished data).

To test the properties of RM40 in Xer site-specific recombination, pSD115, a reporter plasmid containing two directly repeated *cer* sites (Figure 2), was introduced by transformation, and 25–30 pooled colonies were grown at 37°C in 40 ml of medium containing 1.0% glucose. At mid-logarithmic phase, the culture was divided and IPTG (to 2 mM) was added to one of the cultures. Aliquots of each

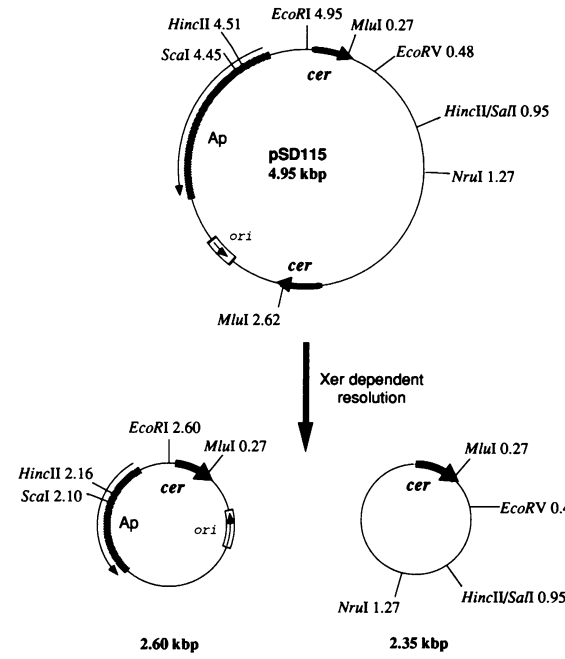


Fig. 2. Diagrammatic representation of Xer-dependent resolution of the *cer* reporter plasmid pSD115. pSD115 is resolved in Xer$^+$ strains to yield 2.6 kbp and 2.35 kbp, circular products. Note that only the 2.6 kbp product contains a replication origin, and hence the smaller recombination product is rapidly lost from bacterial cultures.

culture were taken at the point of IPTG addition and at 30 min time points thereafter and used to prepare plasmid DNA, which was visualized by agarose gel electrophoresis before and after restriction endonuclease digestion (Figure 3).

In the absence of IPTG induction no detectable recombination of pSD115 substrate was observed (Figure 3A and B, lanes 1–5), showing that repression of *xerC* expression from *lacOP* is efficient in the presence of glucose and absence of IPTG. IPTG-induced *xerC* expression led to the resolution of pSD115 (Figure 3A and B, lanes 6–10).

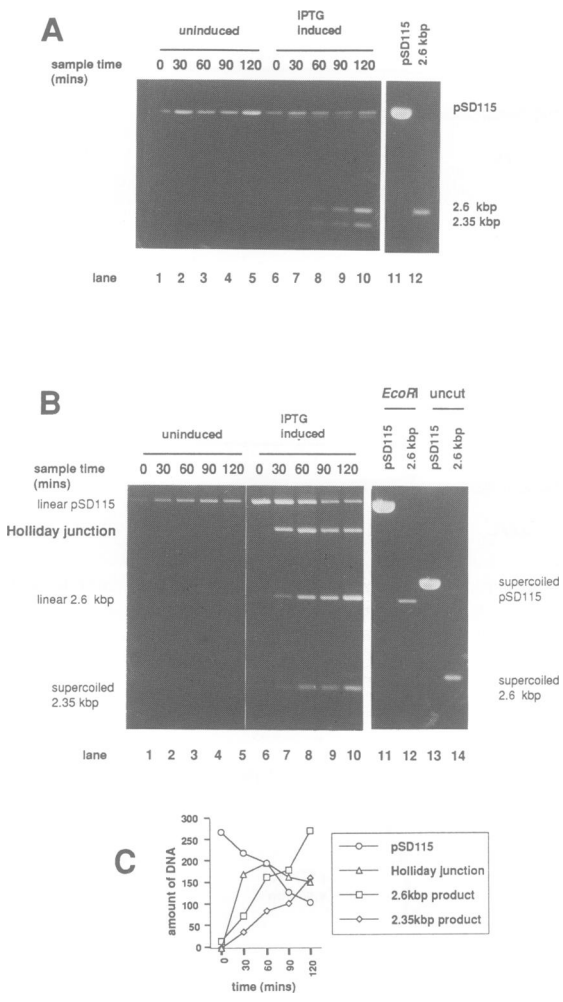


Fig. 3. Time course of *cer* recombination *in vivo* using strain RM40. Two *in vivo* reactions were set up using pooled colonies of RM40 transformed with pSD115: in one (lanes 6–10) *xerC* expression was induced by addition of IPTG at mid-logarithmic phase; in the other (lanes 1–5) the cells were grown in medium containing 1% glucose and *xerC* expression was not induced. In (**A**) the DNA samples were electrophoresed without restriction digestion, and in (**B**) the DNA samples were subjected to digestion with *Eco*RI. Sample times refer to the times at which plasmid DNA samples were prepared after the addition of IPTG and equivalent times in the uninduced samples. DNA was isolated by boiling preparations (including phenol/chloroform extraction) and was run on 1.2% agarose gels. Xer-mediated resolution of pSD115 yields a 2.6 kbp, replicative product and a 2.35 kbp, non-replicative product (which is not observed in standard *cer* recombination assays; lane 12). A single *Eco*RI recognition site is present in pSD115 and in the 2.6 kbp, replicative product (see Figure 2 and lanes 11–14); the 2.35 kbp product contains no *Eco*RI sites. The putative Holliday junction-containing molecules are indicated. A profile of the changing amounts of the four DNA species seen during this assay is shown in (**C**); this was determined by densitometric scanning of the gel shown in (**B**).

Two product circles of the expected 2.6 kbp and 2.35 kbp sizes were seen to increase gradually in quantity after induction, indicating that Xer-mediated recombination at *cer* continued throughout the 2 h time course of the assay. The smaller of these products contains no origin of replication and has not been seen in previous *in vivo* recombination assays using standard Xer$^+$ strains, presumably because it is rapidly diluted out (R.McCulloch and S.Colloms, unpublished). In addition to the generation of product circles on induction of *xerC* expression, we noted that the initial

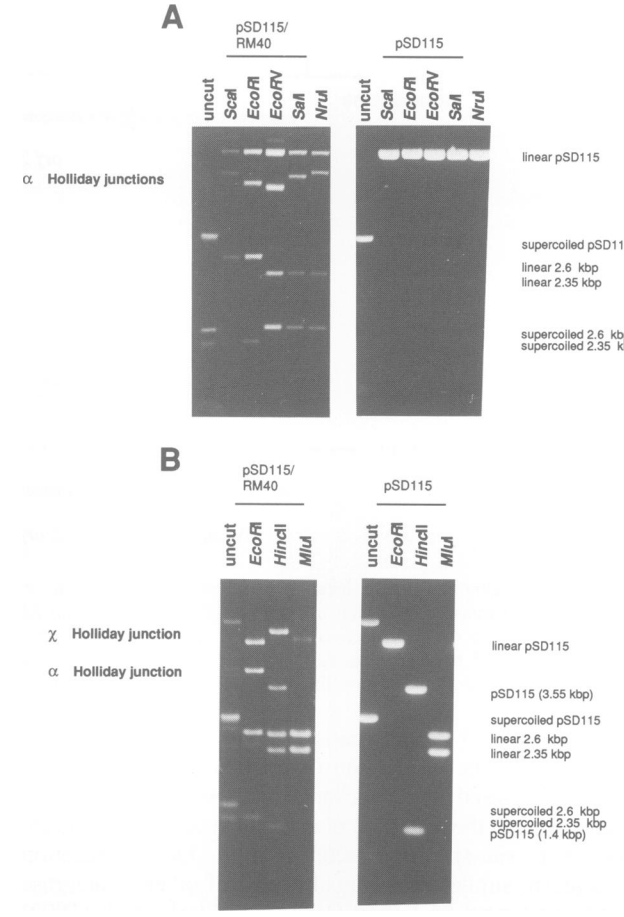


Fig. 4. Analysis of the electrophoretic mobility of pSD115-derived Holliday junction structures after restriction digestion. RM40 transformed with pSD115 was grown to mid-log phase and *xerC* expression induced. After 90 min, plasmid DNA was prepared and digested with either *Sca*I, *Eco*RI, *Eco*RV, *Sal*I or *Nru*I (**A**), or with *Eco*RI, *Hin*cII or *Mlu*I (**B**), before being separated on a 1.2% agarose gel. The positions of the restriction enzyme recognition sequences in pSD115 and how they are distributed into the Xer-mediated 2.6 and 2.35 kbp resolution products is detailed in Figure 2. Supercoiled and linear substrate and product molecules are indicated, as are the putative α structure Holliday junctions generated by single cleavage of the Holliday junctions (A) and the putative χ molecules created by cleavage of both supercoiled domains in the Holliday junction (B).

pSD115 supercoiled band became a doublet that contained a slightly faster migrating species (Figure 3A, lanes 6–10). After digestion with *Eco*RI (which cuts supercoiled pSD115 once), this new DNA species was converted to a form with an electrophoretic mobility intermediate between supercoiled and linear pSD115 (Figure 3B, lanes 6–10); note that the larger (2.6 kbp) recombinant product was linearized by *Eco*RI digestion, while the smaller circle was not cleaved. This digestion also shows that the novel DNA species is only present after the induction of *xerC* expression and is therefore Xer recombination-derived.

The different electrophoretic mobilities of this novel DNA species before and after *Eco*RI digestion can be explained by proposing that a Holliday junction, or a DNA structure with similar physical characteristics, is generated within pSD115. A Holliday junction formed between the two *cer* sites of pSD115 would be confined to the 312 bp of homologous *cer* sequence, since the DNA between these sites is non-homologous pBR322 sequence. This would therefore

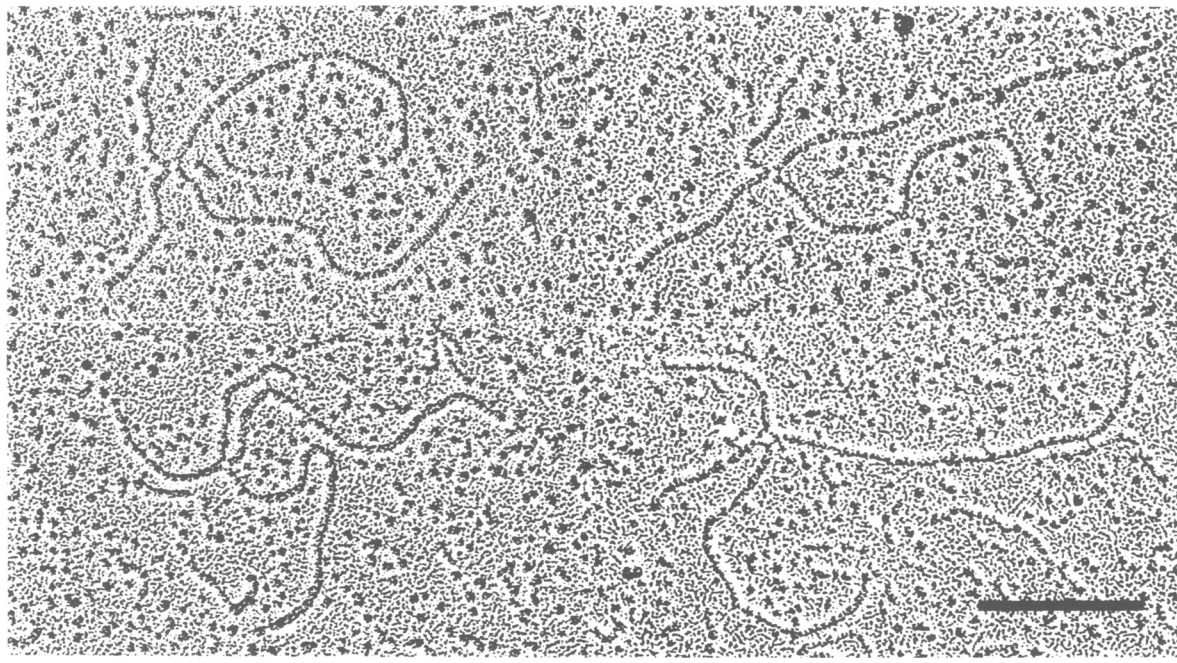


Fig. 5. Electron micrographs of isolated χ structures. A sample of pSD115 DNA isolated after 90 min recombination in RM40 was digested with *Hin*cII and separated on a 1.2% agarose gel (see Figure 4B). DNA was purified from the band representing the *Hin*cII χ structure and spread, in 40% formamide, onto grids and shadowed with platinum:palladium for electron microscopy. All molecules are shown at the same magnification and the bar represents 0.33 μm, which corresponds to ~1 kbp of DNA.

explain the DNA's electrophoretic mobilities, since the uncut plasmid would be a structure resembling a figure 8 that contains two supercoiled domains (2.6 and 2.35 kbp in size) and *Eco*RI digestion would convert the supercoiled molecule into an 'α' structure containing one supercoiled domain and two linear 'arms'.

Electrophoretic analysis of the putative Holliday junction-containing molecules

To confirm that the novel DNA species, produced during Xer site-specific recombination in RM40, contains a Holliday junction, further restriction endonuclease digestions were performed. We hypothesized that different conformations of putative Holliday junction-containing moleclues would be generated by digestion with different enzymes, and that this would be reflected in different relative gel mobilities. Cleavage of either supercoiled domain, using enzymes which cut pSD115 only once (see Figure 2), would be expected to create α structures of varying conformations depending on the position of cleavage; in contrast, cleavage of both supercoiled domains would be expected to generate 'χ' structures. This analysis is shown in Figure 4 and used pSD115 DNA isolated from RM40 after 90 min of IPTG-induced *xerC* expression.

Digestion with all of the enzymes which cleave pSD115 once, generated molecules that were defined as α structures (Figure 4A), on the basis that all their electrophoretic mobilities were intermediate between linear and supercoiled substrate DNA. Although the mobilities of the α molecules might have been expected to simply be a consequence of the size of their supercoiled domains (i.e. 2.35 kbp after digestion with *Sca*I and *Eco*RI, and 2.6 kbp with *Eco*RV, *Sal*I and *Nru*I), this was not the case. It therefore seems likely that the 'arms' extending from the structures contribute to their electrophoretic mobility. It appears that if one arm is very long and the other very short the molecules migrate more quickly through the gel than when the arms are more equally sized. An illustration of this can be seen by comparing the α molecules produced by *Eco*RV, *Sal*I and *Nru*I digestion; these enzymes cleave progressively further from the 'top' *cer* site in pSD115 (174, 640 and 961 bp respectively; Figure 2) and created α molecules with decreasing gel mobilities. Similarly, the *Sca*I α migrated more slowly in the gel than the *Eco*RI α; *Sca*I cleaves 515 bp further from the top *cer* site than *Eco*RI.

Figure 4B shows the results of digestion of the same DNA with *Hin*cII and *Mlu*I, both of which cut twice within pSD115. *Hin*cII digestion linearized both of the resolution products and cleaved pSD115 into 3.55 and 1.4 kbp fragments, as well as converting the putative Holliday junctions into a structure with a lower mobility than both linear pSD115 and the α structures. This is consistent with cleavage in both of the supercoiled domains resulting in a χ molecule. In contrast, *Mlu*I digestion created only product-sized, linear DNA fragments and a small amount of linear substrate (which arose through incomplete digestion). These linear products are generated by cleavage of both circular resolution products and by *Mlu*I digestion within the *cer* sequences of the pSD115 substrate. The lack of χ structures in this digest is most readily explained by proposing that *Mlu*I digestion of the Holliday junction-containing molecules initially creates a χ, but branch migration of the junction within the *cer* homology region moves it to the *Mlu*I ends and releases product-sized, linear DNA fragments. These results therefore suggest that the putative Holliday junction is contained within the *cer* sites of pSD115.

It should be noted that all of the DNA samples analysed above were subjected to phenol/chloroform extraction to remove protein before they were separated by gel electrophoresis. Consequently the possibility that these DNA species may represent protein-bound structures (e.g. synapsed *cer* sites) can be excluded.

Electron microscopy of isolated χ structures

To determine whether our structural predictions based on the electrophoretic characterization of the putative Holliday junction-containing molecules were correct, we used electron microscopy (EM) to visualize gel-purified χ molecules (Figure 5). These were obtained from a sample of *Hin*cII-digested pSD115 DNA isolated from RM40 after 90 min of Xer-mediated recombination (see e.g. Figure 4B). The great majority of the DNA moleules seen in the EM preparation were χ-shaped structures with a central Holliday junction. Thinner strands, representing single-stranded DNA, were observed where the four DNA duplex arms exchange partners. This is probably a result of the DNA duplexes flanking the junction becoming slightly denatured by formamide present in the spreading solution used for the EM.

Arm contour lengths were measured for 36 χ structures in which the position of the Holliday junction could be unambiguously assigned and where the path length of each arm could be accurately determined. Each molecule had two long and two short arms, and within each of these classes there was a longer and a shorter arm. The four arm lengths of each molecule were ranked in order of size, summed, and their lengths as a percentage of the total calculated to be 40.6% (range 35.6−45.1%), 32.7% (28.6−36.6%), 14.3% (12.6−17.5%) and 11.8% (9.3−14.5%). Thus the sizes of the arms of the molecules, assuming a total size for pSD115 of 4950 bp, are 2010 bp (range 1762−2232 bp), 1619 bp (1416−1812 bp), 708 bp (624−866 bp) and 584 bp (460−718 bp). These lengths agree well with the predicted arm lengths of 1990, 1565, 773 and 620 bp, which are based on the positions of the *Hin*cII sites within pSD115 and assume that the Holliday junction is centred on the *cer* sites. The wide range of measured sizes (averaging approximately ± 170 bp from the mean for the four arms) was particularly apparent for the short arms, but there was no unusually large variation in the overall length of the spread DNA molecules. This appeared to be a consequence of reciprocal variations in the lengths of pairs of arms, such that increases and decreases in the length of the longest arm were associated with the same length variations in the longer short arm and the opposite variations in the smaller long arm and the smaller short arm. This is consistent with branch migration of the Holliday junction throughout the 312 bp of *cer* homology, but not into the surrounding, non-homologous plasmid sequence. These data reinforce our view that a major DNA species produced during Xer-mediated site-specific recombination at *cer in vivo* contains a Holliday junction within, or close to, the *cer* sites of pSD115.

Holliday junction-containing molecules have a specific pair of DNA strands exchanged

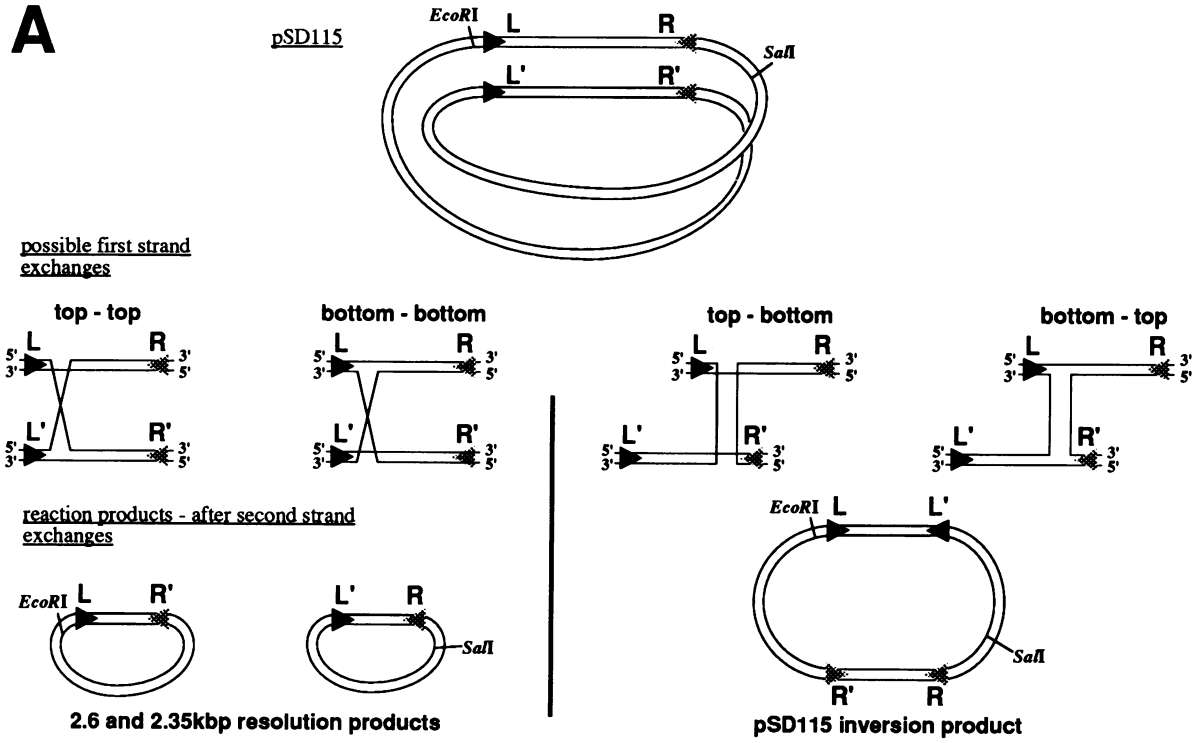
Four combinations of strand exchanges are possible in the formation of Holliday junctions during a site-specific recombination reaction between two DNA duplexes (Figure 6A). Different pairs of second strand exchanges acting on these Holliday junctions are needed to create reaction products (discussed below). We examined the structure of isolated junctions to determine if they had been formed by the exchange of one of these four possible combinations of first strand exchanges. In Figure 6A the *cer* sites are depicted as being aligned in parallel during the strand exchange reaction. It should be noted, however, that we have no experimental evidence regarding this alignment, and this has been done simply to clarify the terms 'top strand' and 'bottom strand' where they are discussed. Note also that the exact positions of strand cleavage and exchange within the *cer* site are unknown, although they are shown arbitrarily towards the left of the *cer* crossover site; this does not alter the interpretation of the experimental results described below.

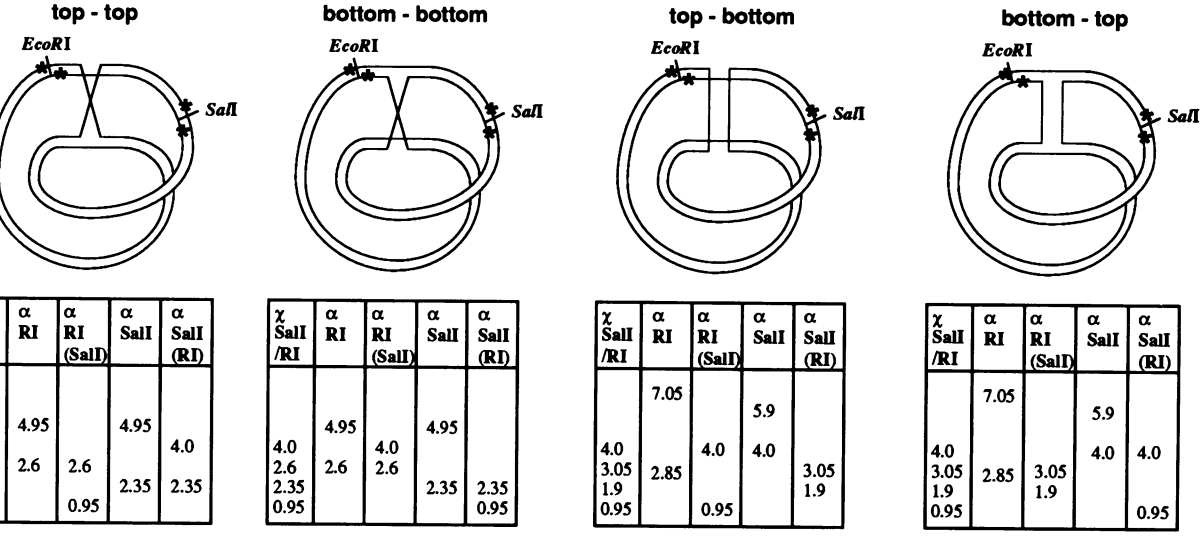
First strand exchanges made between either the top strands or the bottom strands of the recombining duplexes result in Holliday junctions that are resolved to yield recombinants by a second pair of exchanges between the two bottom or two top strands respectively. In either case, recombination between the directly repeated *cer* sites of pSD115 results in resolution of the substrate molecule to give product circles of 2.6 kbp and 2.35 kbp (Figure 6A). Holliday junctions created in either of these ways would be indistinguishable by the electrophoretic and microscopic experiments described above.

Holliday junctions can also be formed by exchanging the top and bottom, or bottom and top, strands of the recombining duplexes (Figure 6A). Resolution of these junctions would be by bottom−top or top−bottom second strand exchanges respectively, and would fuse the left side of one recombining site to the left side of the recombining partner and, reciprocally, right side to right side. This would result in inversion of the DNA between the *cer* sites of pSD115. Such a mechanism is unlikely, because experiments have shown that recombination of directly repeated *cer* sites always results in resolution, and never inversion (S.Colloms, unpublished) and because the *cer* sites would be genetically altered by this reaction. Nevertheless, it was important to exclude this possibility since it is not clear whether their existence can be excluded on this basis or on the grounds of the experiments described above. For example, it is conceivable that the orientation of the *cer* sites is not detected during Xer recombination until after the formation of a Holliday junction, and in the conditions employed here such junctions accumulate before reversal of the first strand exchanges.

To perform this analysis, samples of pSD115 DNA prepared from RM40 after 90 min of recombination were digested with either *Eco*RI, *Sal*I or both *Eco*RI and *Sal*I. Half the restriction digestions were separated on an agarose gel and the other halves were radioactively end-labelled. As expected, *Eco*RI and *Sal*I digestion created α structure Holliday junctions, while the double digestion created χ structure Holliday junctions analogous to those seen by *Hin*cII digestion (Figure 4B).

The *Eco*RI-digested DNA was radioactively end-labelled using [α-^{32}P]dATP, while the *Sal*I and double digestions were end-labelled using both [α-^{32}P]dATP and [α-^{32}P]dCTP. When the Holliday junctions within these digests are labelled in this way, different sizes of radiolabelled single-stranded DNA molecules are predicted for the four possible Holliday junction-containing molecules, as described in Figure 6B. To determine which of these junctions was present, the end-labelled DNA samples were run on a 1.2% low melting point agarose gel and were visualized by autoradiography of the wet gel. DNA from the α structure Holliday junctions in the *Eco*RI and *Sal*I digests, and from the χ structures in the double digest, were purified from isolated gel fragments. Half of the purified *Eco*RI α DNA was digested with *Sal*I, and half of the *Sal*I α was digested with *Eco*RI. These





χ SalI /RI	α RI	α RI (SalI)	α SalI	α SalI (RI)
	4.95		4.95	
4.0				4.0
2.6	2.6	2.6		
2.35			2.35	2.35
0.95		0.95		

χ SalI /RI	α RI	α RI (SalI)	α SalI	α SalI (RI)
	4.95		4.95	
4.0		4.0		
2.6	2.6	2.6		
2.35			2.35	2.35
0.95				0.95

χ SalI /RI	α RI	α RI (SalI)	α SalI	α SalI (RI)
	7.05		5.9	
4.0		4.0	4.0	
3.05	2.85			3.05
1.9				1.9
0.95		0.95		

χ SalI /RI	α RI	α RI (SalI)	α SalI	α SalI (RI)
	7.05		5.9	
4.0			4.0	4.0
3.05	2.85	3.05		
1.9		1.9		
0.95				0.95

Fig. 6. (**A**) Diagram of the possible strand exchange combinations in the formation of Holliday junctions. The upper diagram shows the *cer* sites of pSD115 aligned in parallel before strand exchange. The crossover regions are represented by inverted arrows; the left and right arms of the crossover are denoted by L and L′, and R and R′ respectively (we believe that the left arm is the XerC-binding site and the right arm the XerD-binding site). Four pairs of strand exchanges that would generate Holliday junctions are theoretically possible (as shown). Reaction products that would be generated by a second pair of strand exchanges (see text) on these structures are detailed; note that the two junctions to the left of the diagram are reacted to yield circular resolution products by these exchanges, whilst the junctions to the right are reacted to cause an inverted derivative of pSD115 substrate DNA. (**B**) Single strand size predictions for the four possible Holliday junctions. The positions of incorporation of ^{32}P-labelled nucleotides into *Eco*RI- and *Sal*I-digested DNA that has been end-labelled using the Klenow fragment of *E.coli* DNA polymerase I are represented by an asterisk. The predicted sizes of the end-labelled, single strands (in kb) in the four possible junctions are tabulated for both the χ and α structures (before and after further restriction digestion by the enzymes detailed in brackets). In this diagram RI = *Eco*RI.

samples were then separated, along with undigested *Sal*I α, *Eco*RI α and *Sal*I/*Eco*RI χ, on a denaturing agarose gel and the single-stranded DNA was visualized by autoradiography after vacuum drying. BRL 1 kbp marker DNA (visualized by ethidium bromide staining) was included on the same gel to allow the sizes of the radioactive single-strands to be determined. The result of this experiment is shown in Figure 7.

The sizes of the radiolabelled single-stranded DNA in the uncut samples (Figure 7, lanes 1, 3 and 5) showed that the

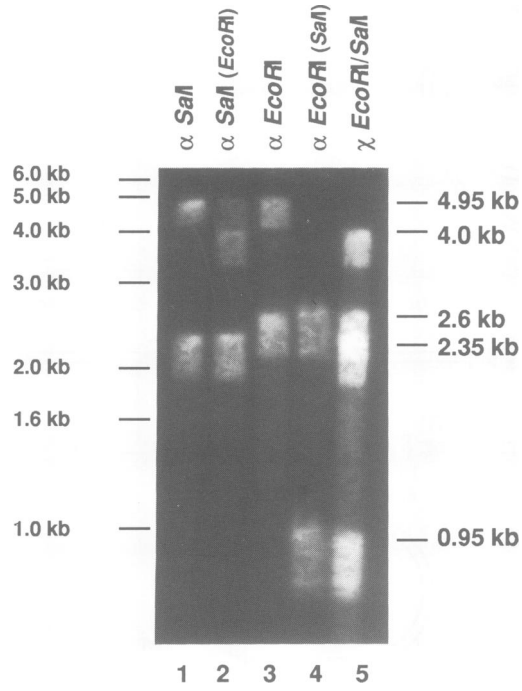


Fig. 7. Asymmetry in the first pair of *cer* strand exchanges. Plasmid DNA representing the reporter plasmid pSD115 after 90 min of Xer-mediated recombination in RM40 was digested with either *Eco*RI, *Sal*I or both *Eco*RI and *Sal*I. Half the reaction volumes were radioactively end-labelled, separated on a 1.2% Seaplaque low melting point agarose gel and the DNA visualized by autoradiography of the undried gel. The *Eco*RI α, *Sal*I α and *Eco*RI−*Sal*I χ Holliday junction molecules were then purified and separated, before or after further restriction digestion, on a 1.2% alkaline, denaturing agarose gel and the DNA visualized by autoradiography after vacuum drying. The sizes of the radiolabelled single strands are shown (they were derived from 1 kbp BRL size markers run on the same gel), and the predicted sizes for the four possible Holliday junctions are detailed in Figure 6B. The lane order was as follows: lane 1, *Sal*I α (undigested); lane 2, *Sal*I α (*Eco*RI-digested); lane 3, *Eco*RI α (undigested); lane 4, *Eco*RI α (*Sal*I-digested); lane 5, *Eco*RI−*Sal*I χ (undigested).

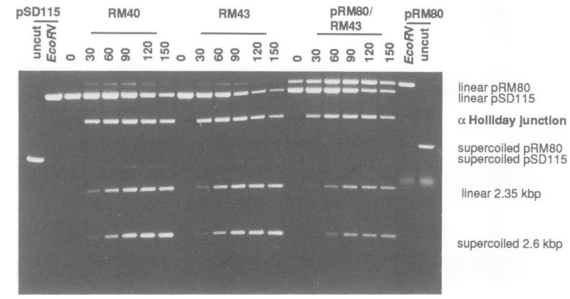


Fig. 8. *In vivo* recombination of pSD115 in RuvC$^+$ and RuvC$^-$ derivatives of RM40 over 150 min. *In vivo* recombination assays were performed using pSD115 transformed into the following strains: RM40, RM43 and RM40 containing pRM80. Plasmid DNA samples were prepared by the boiling method at the times shown after IPTG induction of *xerC* expression and were digested with *Eco*RV before separation on a 1.2% agarose gel. *Eco*RV digestion linearizes the RuvC expression vector pRM80 and also linearizes pSD115 and its 2.35 kbp recombinant product (see Figure 2). α structures corresponding to the Holliday junctions are indicated (note that the DNA species migrating more slowly than linear pSD115 in the DNA samples prepared from strains RM40 and RM43 is the relaxed form of the α Holliday junction).

Holliday junctions resulted from either top−top or bottom−bottom first strand exchanges, and not from top−bottom or bottom−top (compare the sizes in Figure 7 with the predictions in Figure 6B). This suggests that the Holliday junctions in these assays have not arisen through aberrant 'inversion' fusions between directly repeated *cer* sites. Top and bottom first strand exchanges were distinguished by *Sal*I digestion of *Eco*RI α DNA and *Eco*RI digestion of *Sal*I α DNA (Figure 7, lanes 2 and 4). The radiolabelled single strands produced by these restriction digestions were 2.6 kb and 0.95 kb, and 4.0 kb and 2.35 kb respectively, suggesting that the top strands had been specifically exchanged to create these Holliday junctions. Even on longer exposures of this and similar gels the fragment sizes that would indicate junctions derived from bottom strand exchanges were not observed (data not shown).

Xer-mediated recombination at cer in argR and pepA derivatives of RM40, and in the presence and absence of RuvC

Xer-mediated site-specific recombination at *cer* has an absolute requirement for the accessory proteins ArgR and PepA (Stirling *et al.*, 1988b, 1989). In order to determine if these proteins are required for Holliday junction formation in RM40, *argR* and *pepA* derivatives of this strain (RM41 and RM42 respectively) were constructed. Neither Holliday junction-containing molecules nor recombinant products were observed in recombination assays using pSD115 in RM41 and RM42 (data not shown), confirming previous demonstrations of the requirement for these proteins in Xer recombination at *cer*. The lack of Holliday junctions may indicate that ArgR and PepA function at a stage in the Xer reaction prior to the strand exchanges which generate the Holliday junctions, for example in synapsis of *cer* sites (Summers, 1989; Blakely *et al.*, 1993). However, the possibility cannot be excluded that Holliday junctions are formed in RM41 and RM42 but are more rapidly converted back to substrate than in ArgR$^+$ and PepA$^+$ strains.

In an attempt to determine whether the 2.6 kbp and 2.35 kbp circular products of recombination seen in RM40 after induction of *xerC* expression are derived totally by Xer-catalysed recombination, or are a result of cellular enzymes processing the Holliday junction-containing molecules, we assayed pSD115 recombination in a *ruvC* derivative of RM40 (RM43) and in an RM40 derivative expressing *ruvC* from a multicopy plasmid (pRM80). RuvC is the best characterized *E.coli* protein that can process Holliday junctions (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991; Takahagi *et al.*, 1991). Neither the absence of RuvC, nor its presumed expression from pRM80, significantly altered the pattern of pSD115 recombination in comparison with RM40 (Figure 8); in all the strains Holliday junction-containing molecules and recombinant products were present in comparable quantities and were generated at similar times during the assay. These results indicate that RuvC does not play a major role in processing the observed Holliday junctions, even though we have demonstrated that RuvC is capable of cleaving pSD115 Holliday junction-containing molecules *in vitro* (R.McCulloch, H.Dunderdale and S.West, unpublished).

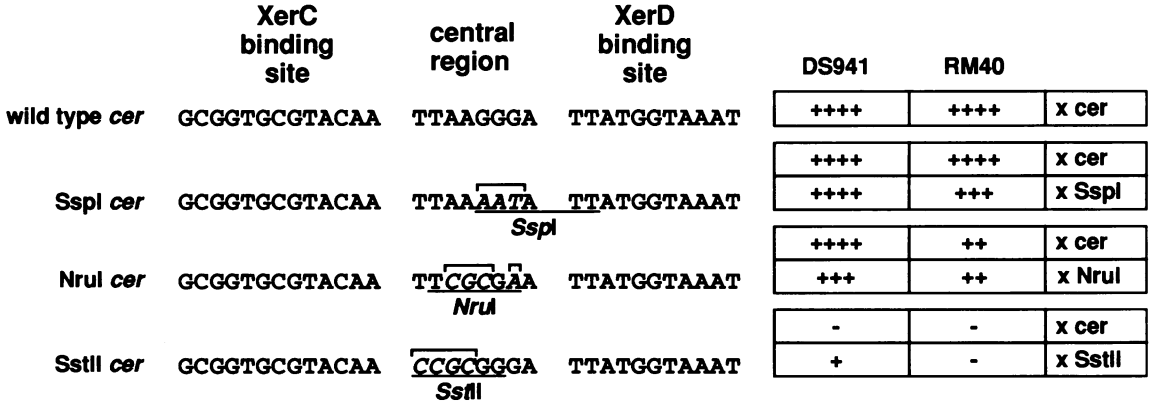


Fig. 9. Recombination efficiencies of Xer-mediated reactions involving *cer* site variants containing sequence alterations in their central regions. The crossover regions of three *cer* variants containing sequence alterations to their central regions are shown, and the XerC and XerD binding sequences of these sites are indicated. Heterologous sequence in comparison with wild type *cer* is identified by italics and by lines over the sites; the restriction enzyme recognition sequences which identify the sites are underlined. Recombination efficiency of the sites both in reactions against wild type *cer* (× *cer*) and against themselves (× self) is detailed semi-quantitatively; this was determined by estimating the extent that reporter plasmid substrates containing the sites were converted to recombinant products (including Holliday junctions) over a 2 h time course in RM40 and over 30 generations in DS941. '++++' represents the extent that a reporter plasmid with two wild type *cer* sites is converted to product (100% in DS941, and ~60% in RM40; see Figure 3). '+++' represents a small reduction (10–15%) in the extent of substrate conversion. '++' represents a 50–60% reduction in the extent of substrate conversion. '+' represents only a small amount (~10–15%) of substrate conversion. '–' represents no detectable recombination of the reporter plasmid.

Effects of central region sequence heterology on the efficiency of Xer-mediated recombination at cer

In the characterized integrase-family recombination systems, the points of strand cleavage and exchange within the crossover regions of the recombination sites have been shown to be at fixed staggered positions on the top and bottom strands of the recombining DNA duplexes (Craig and Nash, 1983; Hoess and Abremski, 1985; McLeod *et al.*, 1986). The DNA sequence between these cleavage points has been called the overlap or spacer region and varies in size from 6 to 8 bp in the different recombination sites. In all sites, the overlap region is flanked by binding sequences for the cognate recombinases. Extensive analysis has suggested that the sequence of the overlap is relatively unimportant, since mutations within the region are often functional when paired with other sites containing the same sequence changes. Sequence homology in the overlaps of recombining sites is important, however, since recombination between sites with heterologous overlap regions is inefficient and can lead to the production of altered recombination products (Weisberg *et al.*, 1983; Bauer *et al.*, 1985; Senecoff and Cox, 1986; Andrews *et al.*, 1987; Hoess *et al.*, 1987; Kitts and Nash, 1987; Nunes-Duby *et al.*, 1987; de Massy *et al.*, 1989).

The exact positions of strand cleavage within *cer* are not yet known. However, sequence comparisons between *cer* and related sites (Sherratt *et al.*, 1993) and analysis of XerC and XerD binding to *dif* and *cer* (Blakely *et al.*, 1993) suggests that the central region of *cer* (Figure 9) may correspond to the overlap region of core sites of other integrase family members. In an attempt to determine the function of the *cer* central region three *cer* variants were generated with sequence alterations that span 7 bp of the 8 bp central region and which therefore should create heterologies during recombination with wild type *cer*. These variants bound XerC and XerD *in vitro* (data not shown). The effect these changes have on Xer recombination efficiency was analysed by constructing a series of reporter plasmids equivalent to pSD115 but containing wild type *cer* and/or the variant sites. Recombination was analysed in two ways, (i) by examining plasmid recombination over a 2 h time course in strain RM40, and (ii) introducing the plasmids into a standard Xer$^+$ strain, DS941, and examining the DNA ~30 generations later. The results of this analysis are summarized in Figure 9.

Recombination between wild type *cer* and the two *cer* variants, *Ssp*I *cer* and *Nru*I *cer*, was as efficient as recombination between the variant sites themselves (in fact, in DS941, the *Nru*I *cer* variant was poorer when paired with itself than with wild type *cer*). In addition, the amount of Holliday junctions derived from the substrates containing the variant sites in the RM40 assays was reduced by the same extent as the reduction in the amounts of complete recombinant products (data not shown). This indicates that the sequence heterologies between these sites and wild type *cer* do not cause increased Holliday junction accumulation, nor does altering the central region in these ways overcome the Holliday junction accumulation in RM40 recombination assays.

No recombinant products (including Holliday junctions) were seen during recombination between *cer* and *Sst*II *cer*. Recombination between two *Sst*II *cer* variants was highly inefficient, but detectable, over 30 generations in DS941. Taken with the above data on recombination between matched *cer* variants, it is apparent that the sites are progressively poorer recombination substrates when sequence changes move towards the XerC binding site. If the lack of recombination between *Sst*II *cer* and *cer* is a consequence of the 4 bp sequence heterology in the central region, then the 2 bp CC dinucleotide adjacent to the XerC binding site is most likely the cause, because the other 2 bp of non-homology overlap with the heterology between *Nru*I *cer* and wild type *cer*. We do not know if the inefficient recombination of the *Sst*II *cer* variant with itself is a consequence of altered XerC/D binding or reduced cleavage.

We note that natural *cer* homologues with 8 bp central regions all contain TT as the first two nucleotides (Sherratt *et al.*, 1993) rather than the CC of the *Sst*II variant.

Discussion

We originally devised a system to analyse the Xer site-specific recombination reaction *in vivo* because of our failure to observe the reaction *in vitro*. A strain, RM40, was constructed in which the expression of *xerC* from its chromosomal locus could be tightly regulated. *xerC* was chosen for this purpose because at the outset of this work we believed it to be the sole Xer recombinase (Colloms *et al.*, 1990). Subsequently, however, we have identified a second recombinase, XerD, whose wild type gene is present in RM40 (Blakely *et al.*, 1993).

The recombination substrate employed in the experiments described is a plasmid (pSD115) containing two directly repeated *cer* sites. In the absence of induction, pSD115 could be maintained in RM40 with little or no Xer-mediated resolution (typically $<10\%$ was observed after 30–40 generations of uninduced growth). After IPTG-induced *xerC* expression, products consistent with intramolecular resolution appeared rapidly and increased over a 2 h time period. We saw no evidence for intermolecular recombination. The pattern of appearance of Holliday junction-containing molecules and complete recombinant products is consistent with a precursor–product relationship between the two (Figure 3). After 60 min of *xerC* expression, $\sim 30\%$ of the total plasmid DNA and $\sim 24\%$ of plasmid molecules contain Holliday junctions. After overnight *xerC* induction on solid medium resolution of pSD115 is complete in RM40, with all of the observable plasmid DNA being the 2.6 kbp product (data not shown). After extended *xerC* expression, therefore, the profile of recombinant products is indistinguishable from that seen in a normal XerC$^+$ XerD$^+$ strain. Generation of any detectable product was dependent on the presence of the accessory proteins ArgR and PepA. The conclusion that these molecules contain Holliday junctions within the homologous *cer* sequences of pSD115 is substantiated by the demonstration (unpublished data of R.McCulloch, D.Sherratt and S.West) that the supercoiled structures are substrates *in vitro* for purified RuvC, an *E.coli* enzyme that cleaves four-way DNA junctions containing a region of sequence homology (Connolly *et al.*, 1991; Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991; Takahagi *et al.*, 1991).

Holliday junctions are believed to be intermediates in the site-specific recombination reactions catalysed by the λ integrase family of recombinases [for reviews see Landy (1989) and Stark *et al.* (1992)]. Their isolation has previously been achieved in *in vitro* recombination reactions using altered recombination sites, mutant proteins or specific reaction conditions (Hoess *et al.*, 1987; Nunes-Duby *et al.*, 1987; Jayaram *et al.*, 1988; Kitts and Nash, 1988a; Meyer-Leon *et al.*, 1988, 1990). In addition, there is both indirect and direct evidence for the existence of Holliday intermediates during *in vivo* λ Int-catalysed recombination (Valenzuela and Inman, 1975; Echols and Green, 1979; Enquist *et al.*, 1979). Molecules containing Holliday junctions have also been observed during Xer-mediated intramolecular recombination *in vivo* between ColE1 *cer* and the related site of CloDF13 (*parB*; Hakkart *et al.*, 1984) in wild type Xer$^+$ strains (G.Sullivan and D.Summers, personal communication). The detection of substantial quantities of Holliday junction-containing molecules during Xer-mediated recombination at *cer* using wild type proteins and sites supports the prediction, based on recombinase homology (Colloms *et al.*, 1990; Lovett and Kolodner, 1991; Blakely *et al.*, 1993), that the Xer strand exchange mechanism is similar or identical to that of λ Int and its relatives.

Our ability to detect Holliday junctions so readily and in such large quantities is surprising, since they are believed to be transient reaction intermediates in other systems and only accumulate when their conversion to products or reversion to substrate is prevented. This may therefore suggest that there is something unusual about the *cer* site-specific recombination reaction when it is compared with other λ integrase-like reactions. For example, no Holliday junctions were reported in the similar *in vivo* analyses performed on either λ Int- or Cre-catalysed reactions (Bliska and Cozzarelli, 1987; Adams *et al.*, 1992). An important question which therefore arises is whether this accumulation of Holliday junctions is a normal property of Xer site-specific recombination at *cer*, or whether it is a feature confined to the strain RM40. We have not observed Holliday junctions in recombination assays in wild type cells, though in these assays plasmid DNA was not prepared until 20–30 generations after substrate DNA had been introduced. For these reasons we employed several strategies in an attempt to exclude potential sources of experimental artefact in our *in vivo* analysis.

In RM40, the genes encoding DapF and ORF235 have been mutated by insertions. Since it is possible that these proteins have a role in the Xer recombination reaction that we do not yet understand, we created a strain, RM50, in which the entire *xerC*-containing transcriptional unit is functionally intact and is controlled by *lacPO*. Holliday junctions were detectable in comparable quantities when RM50 was used in time course assays in the same manner as RM40 (data not shown), suggesting that the inactivation of *dapF* and *orf235* is not the cause of Holliday junction accumulation.

We also considered whether *xerC* or other genes (e.g. *xerD*) in RM40 carried single or multiple mutations responsible for the observed Holliday junction accumulation. For example, it is known that in Cre recombination *in vitro*, certain derivatives of the recombinase cause the appearance of Holliday intermediates that are not detectable when wild type protein is used (Hoess *et al.*, 1987). We therefore constructed a plasmid, pRM60, in which the expression of *xerC* is from the λP_L promoter and is controlled by the cI857 repressor. The use of this plasmid in *in vivo* assays, which is comparable to the approach adopted by Bliska *et al.* (1991), again led to the appearance of Holliday junction-containing molecules during recombination of pSD115 (data not shown). Because the strains used for this experiment have a different origin to RM40, and because the *xerC* gene in the plasmid was cloned independently and from a different source to the gene used in the construction of RM40, we conclude that mutations in *xerC* or other genes cannot account for the observed, stable Holliday junctions. Moreover, the method of repression and activation in pRM60 is quite different to RM40, suggesting that it is not the IPTG induction of *xerC* expression in RM40 that has this effect.

Our standard strains contain a *recF* mutation in order to minimize homologous recombination between and within plasmids. Utilization of pRM60 to promote Xer-mediated recombination of pSD115 in an otherwise XerC$^-$ RecF$^+$ strain (DS8003) allowed us to show that Holliday junction accumulation is not the result of the mutant *recF* allele in RM40 (data not shown).

Since there appears to be no unique genetic defect in RM40 responsible for the Holliday junction accumulation, we tested whether some feature inherent to the reporter plasmid pSD115 had this effect. This could, for example, be the result of undefined alterations of the *cer* sites in the plasmid (perhaps creating site mutations analogous to those that cause junction accumulation in other systems; Nunes-Duby *et al.*, 1987; Kitts and Nash, 1988a), or could be due to the replication or transcription pattern of pSD115, or even some secondary structure the plasmid adopts *in vivo*. To address these possibilities, recombination time courses were performed in RM40 using two other recombination substrates, pCS202 and pKS455, which also contain directly repeated *cer* sites (Stirling *et al.*, 1988b; Colloms *et al.*, 1990). Holliday junctions were clearly visible using these plasmids, despite the fact that their patterns of transcription and replication, and their cellular copy numbers, differ from pSD115 (data not shown). In addition, these plasmids were constructed independently from pSD115 and from different sources, making it extremely unlikely that a common mutation in one or both *cer* sites exists in all plasmids which could be the cause of junction accumulation.

We are unable, at present, to explain why Xer site-specific recombination in RM40 should produce substantial levels of Holliday junctions. It is possible that the stable junctions are a result of the altered expression of *xerC* in RM40 causing changes to the natural relative concentrations of the two recombinases (note that *xerD* had not been identified when this work was initiated). For technical reasons we have not been able to test the effects of varying XerD levels on recombination in cells in which XerC is absent or present at different levels. However, it is not clear why an alteration of cellular XerC levels should cause the reaction to stop after the first pair of strand exchanges and become unable to either progress forward and execute the second pair of strand exchanges, or to reverse the first exchanges and recreate the substrate. This is especially true if our belief that XerC and XerD each catalyse one pair of strand exchange is correct (Blakely *et al.*, 1993). One explanation of the data is that the recombination complex dissociates in RM40 after an XerC-mediated strand exchange. If the resulting Holliday junction branch migrates away from where it is created, its presence could prevent reformation of a recombination complex and block its conversion to recombinant product or back to substrate (perhaps by a similar mechanism to that which normally prevents intermolecular recombination at *cer*). Another possibility is that the Holliday junctions remain bound to the recombinase proteins in some energetically stable state which does not readily allow further recombination. Footprinting experiments *in vivo* might distinguish these possibilities.

An alternative explanation for the stable Holliday junctions is that the recombination reaction at *cer* naturally involves only one pair of strand exchanges and the observations presented here reflect the normal, complete Xer recombination reaction. If this were true, then the complete recombinant products normally seen must be derived from processing of the Holliday junctions by cellular enzymes, though we have not observed an effect of *in vivo* RuvC levels on Holliday junction half-life. The fact that both wild type recombinases contain highly conserved residues implicated in catalysis, and the observation that mutations created within these residues interfere with product formation (Blakely *et al.*, 1993), argue against this, as does the observation that intermediates containing Holliday junctions have not been seen in wild type Xer$^+$ cells.

Isolation of the *cer*-derived Holliday junctions has allowed us to determine that a particular pair of DNA strands (the 'top' strands) within the recombining duplexes have been specifically exchanged during their formation. We infer that the cleavages that give rise to these exchanges occur adjacent to where XerC binds, therefore implicating XerC catalytic activity in this step of the reaction. The reasoning is as follows: in Figure 6A, the left half of the *cer* crossover binds XerC and the right half XerD (Figure 9); if staggered cleavages occur at each end of the *cer* central region with the same polarity as the cleavages characterized in the overlap regions of other λ integrase family reactions, then a top-strand cleavage close to the XerC binding site at the 5′ end of the central region would be expected.

This specificity of strand exchange suggests there is an asymmetry, or bias, in the recombination reaction at *cer*. The asymmetry may reside in either the first strand exchange step of the recombination reaction or in the resolution of the Holliday junction intermediates. Since we have not demonstrated that the stable Holliday junctions in RM40 are resolved by Xer recombination, it is possible that their accumulation is a consequence of the poor resolution of junctions in which the top strands have exchanged; this would mean that the reaction products we detect arise through the rapid resolution of Holliday junctions whose bottom strands were exchanged first. Alternatively, the stable junctions may represent the only intermediates that are created during Xer-mediated recombination at *cer*, and consequently the strand exchange asymmetry resides in their formation, not their resolution. The latter hypothesis is favoured in explaining the strand exchange asymmetry that is observed in λ Int and Cre recombination (see below; Hoess *et al.*, 1987; Kitts and Nash, 1988b). If this were also true for Xer recombination, then the asymmetry must lead to an order in the strand exchanges, with the top strands being exchanged first and the bottom strands second.

We can imagine three potential sources of asymmetry in Xer recombination at *cer*, though we cannot yet be certain which one is the source of strand exchange asymmetry. These are, asymmetry in the XerC and XerD binding sites; asymmetry in the central region; and asymmetry introduced by accessory sequences being on one side of the *cer* recombination core. We know that during Xer recombination at *dif*, it is asymmetry of binding sites (one interacting with XerC and the other with XerD) rather than central region asymmetry that ensures that only correctly aligned sites are recombined (Blakely *et al.*, 1993). However, this does not mean the same mechanism determines strand exchange asymmetry at *cer*. Phage λ Int and Cre use different mechanisms (asymmetry introduced by accessory sequences and asymmetry in the central overlap region respectively) to determine their observed ordered strand exchanges (Hoess *et al.*, 1986, 1987; Kitts and Nash, 1988b).

Xer recombination between wild type *cer* and two of the *cer* variants (*Ssp*I *cer* and *Nru*I *cer*) containing either 3 or 4 bp of heterology in the central region gave surprising results. The heterologies hardly affected the overall yield of recombinant products (including Holliday junctions) when compared with recombination of each of the variants with itself (Figure 9), indicating that Holliday junction formation and resolution is not perturbed by these heterologies. This situation contrasts sharply with that of λ Int and its characterized relatives, where overlap region heterology does not normally interfere with Holliday junction formation, but is thought to block subsequent branch migration, resulting in rapid reversal of the initial pair of strand exchanges (Weisberg *et al.*, 1983; Bauer *et al.*, 1985; Hoess *et al.*, 1986; Senecoff and Cox, 1986; Andrews *et al.*, 1987; Kitts and Nash, 1987; Nunes-Duby *et al.*, 1987). Our results could mean that cleavage and strand exchange do not occur in the *cer* central region. However, we prefer explanations in which Holliday junction accumulation is a consequence of either a rapid dissociation of the recombination complex after the first pair of strand exchanges, with a barrier to further exchanges, or the recombination complex being trapped in a stable structure after the first exchanges. We have found no role for homology testing by branch migration or other means in Holliday junction formation and resolution in recombination at *cer*.

The data presented here provide the beginnings of a mechanistic analysis of the Xer site-specific recombination reaction. Although we do not fully understand the significance of all of the data, recent results from *in vitro* experiments on Xer-mediated recombination at *cer* (our unpublished data) confirm the main experimental features presented here. We believe that the results we observe are, at least in part, a consequence of the Xer system requiring two recombinases; this provides the potential to dissociate one pair of strand exchanges from the other and to have them under separate biochemical control. We wish to determine the precise roles of the recombination proteins in the Xer reaction and whether the accumulation of Holliday junctions is directly related to the roles of Xer site-specific recombination in chromosome segregation and plasmid stability. Finally, the ability to produce large quantities of supercoiled plasmids containing Holliday junctions in strains in which XerC levels are controlled provides a valuable tool for those wishing to use and study Holliday junction-containing molecules.

Materials and methods

Bacterial strain construction

All strains except DS8003 are derivatives of *E.coli* K-12 AB1157 (Bachmann, 1972). DS941 is AB1157 *recF lacI*^{q} *lacZ*ΔM15, DS942 is AB1157 *recF* Δ*lacZ*H220 *lacI*, DS981 is DS941*xerC2* (Colloms *et al.*, 1990). RM40 (described in text) is a derivative of DS941 that was constructed in the following way: pRM102 (see below) was linearized by *Pvu*II digestion and transformed into the strain JC7623 (Winans *et al.*, 1985); a derivative of JC7623 in which the chromosomal *xerC* gene had been replaced by the plasmid-borne gene was selected by virtue of being resistant to kanamycin and sensitive to ampicillin (the plasmid-borne resistance); the *xerC* allele of this derivative (now under the control of the *lac* promoter) was then transduced into DS941. DS8003 is a RecF$^+$ XerC$^-$ derivative of CSH5O (Miller, 1972). RM41 and RM43 were constructed by transduction into RM40 of the *argR* allele from DS941*xerA9*::*fol* (Colloms *et al.*, 1990) and the *ruvC53* allele from CS85 (a gift of R.Lloyd) respectively, while RM42 was made by transduction of the *xerC* gene (and upstream control elements) of RM40 into DS941*pepA7* (Colloms *et al.*, 1990). DS8003 is an *xerC2* derivative of the RecF$^+$ strain CSH50 Δ(*fimB-H*) made by transduction from DS981 (Miller, 1972; Blakely *et al.*, 1991).

Bacteriophage P1*kc* was used for generalized transduction (Miller, 1972) and all plasmid transformations in this work used standard $CaCl_2$ treatment of cells (Cohen and Hsu, 1972; Sambrook *et al.*, 1989).

Bacterial growth media and conditions

Bacteria were grown in L broth (Miller, 1972) at 37°C. Antibiotics, agar and other supplements were added to the medium where appropriate. RM40 cultures on solid medium were supplemented with diaminopimelic acid (50 μg/ml; Sigma; a mixture of LL, DD and *meso* isomers). Glucose was used at 1% to maintain repression of the chromosomal *lac* promoter in RM40.

Plasmid constructions

The reporter plasmid pSD115 contains two directly repeated *cer* sites (Figure 2), both of which were derived from pKS492 [which has been described previously (Summers and Sherratt, 1988)]. It is essentially pBR322, with one *cer* inserted as a 341 bp fragment between the *Eco*RI and *Hin*dIII sites (giving pSD113) and the other one inserted as a 312 bp *Sma*I−*Hin*dIII fragment in the *Pvu*II site. Plasmids containing the *cer* variants are identical to pSD115 other than for their altered central region sequences (Figure 9); these sequences were confirmed by DNA sequencing.

pRM102 is the plasmid that was used to generate the strain RM40 (see above); it was created in two steps from the plasmid pSD102 (Colloms *et al.*, 1990), which contains a 3.6 kbp *E.coli* chromosomal fragment encompassing the genes *dapF*, *orf235*, *xerC* and part of *orf238*. In the first step of constructing pRM102, *lac*PO was inserted into the unique *Nru*I site of pSD102 (96 bp upstream of the *xerC* start codon) as a 230 bp *Pvu*II−*Sma*I fragment derived from pUC19, giving rise to the plasmid pRM101. The *rrnB* transcriptional terminator and Tn*903* kanamycin resistance gene were cloned into pRM101 to create pRM102 as, repectively, a 450 bp *Ssp*I−*Hin*dIII fragment derived from pKK223-3 and a 1.3 kbp *Pst*I fragment from pUC71K (both plasmids obtained from Pharmacia). This was achieved by cloning the fragments sequentially into the polylinker of pIC20R (a gift from R.Wilson), and then inserting them together into *Stu*I-digested pRM101 as a single 1.8 kbp *Eco*RI fragment. As a result of these clonings, 440 bp of operonic sequence has been replaced by control sequences in pRM102 (see Figure 1), therefore deleting the final 180 bp of *dapF* (corresponding to the C-terminal 58 amino acid residues of DapF) and the first 260 bp of *orf235* (the N-terminal 85 residues of ORF235).

pRM80 was constructed by cloning a 1.12 kbp *Hin*dIII−*Eco*RI *ruvC*-containing fragment from pGS762 (Sharples and Lloyd, 1991) into the λ dv-based expression vector pCB105 (a gift of C.Boyd) which had also been digested with *Hin*dIII and *Eco*RI. Transcription of *ruvC* in pRM80 is derived from the plasmid's *lac* promoter, and RuvC expression was confirmed by demonstrating that pRM80 is able to confer UV resistance to the UV-sensitive strain RM43.

pRM60 was constructed as follows, a 1.2 kbp *Hin*dIII−*Eco*RI *xerC*-containing fragment from pSD105 (Colloms *et al.*, 1990) was cloned into *Eco*RV-digested pCT1050 (a gift of R.Thompson), creating the plasmid pRM50 (where *xerC* transcription is derived from the plasmid's λP_L promoter); a 1.6 kbp *Eco*RI−*Bam*HI fragment encompassing both *xerC* and the λP_L promoter sequences was then cloned from pRM50 into *Eco*RI- and *Bam*HI-digested pGP1-2 (Tabor and Richardson, 1985), creating pRM60. In pRM60 the *cI857* gene product represses *xerC* transcription at 28−30°C, but this repression is overcome by elevating the temperature to 37−42°C.

In vitro DNA manipulations

DNA manipulations were performed essentially as described in Sambrook *et al.* (1989). The restriction enzymes, ligase and Klenow fragment of *E.coli* DNA polymerase I used in these experiments were all purchased from BRL; the [α-^{32}P]dATP/dCTP used for end-labelling was purchased from NEN. Plasmid DNA from *in vivo* recombination experiments was purified from cells using either the boiling (STET) method of Holmes and Quigley (1981), or columns purchased from Qiagen (following the instructions supplied). DNA was purified from standard agarose gels for electron microscopy by low speed centrifugation through siliconized glass wool (Heery *et al.*, 1990), and from 'Sea-plaque' low melting point gels for end-labelling by phenol/chloroform extraction (following the instructions supplied by the agarose manufacturers, FMC Bioproducts).

Three kinds of horizontal agarose gels were used, namely standard, low melting point (LMP) and alkaline denaturing. All were 1.2% and electrophoresis was at ~3 V/cm. Standard and LMP gels were made and run in Tris−acetate buffer (40 mM Tris−AcOH pH 8.2, 20 mM NaOAc, 1 mM EDTA), whilst the denaturing gels were made and run in 50 mM NaOH, 1 mM EDTA. DNA was visualized either by 254 nm UV

illumination after staining with ethidium bromide, or by autoradiography. Ethidium bromide-stained gels were photographed onto Ilford HP5 film, and the autoradiographs used Fuji X-ray film (but are shown as negative contact prints on HP5 film). Quantification of DNA from negatives used a Joyce–Loebel densitometer; calculations took into account the differential ethidium bromide binding of supercoiled and linear DNA and the different sizes of molecules.

Electron microscopy

Electron microscopic (EM) examination of purified DNA was performed as described by Coggins (1987). The DNA was spread in 40% formamide, 1 × TE (100 mM Tris pH 8.3, 10 mM EDTA), 0.1 mg/ml cytochrome *c* on a hypophase of 10% formamide, 0.1 × TE and was adsorbed to collodion-coated grids. Contrast was enhanced by staining with uranyl acetate, and low angle (8–10°) shadowing was performed by vacuum evaporation of platinum:palladium (80:20) wire. The DNA used for the preparation of χ structures (and for determination of the exchanged strands) was isolated from a 200 ml culture of RM40 transformed with pSD115 that had been induced at mid-logarithmic phase with IPTG (2 mM) for 90 min. The arm lengths of the molecules were measured from the electron micrographs using a map measurer.

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