Xer-mediated site-specific recombination *in vitro*

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The Xer site-specific recombination system acts at ColE1 *cer* and pSC101 *psi* sites to ensure that these plasmids are in a monomeric state prior to cell division. We show that four proteins, ArgR, PepA, XerC and XerD are necessary and sufficient for recombination between directly repeated *cer* sites on a supercoiled plasmid *in vitro*. Only PepA, XerC and XerD are required for recombination at *psi* *in vitro*. Recombination at *cer* and *psi* *in vitro* requires negative supercoiling and is exclusively intramolecular. Strand exchange at *cer* produces Holliday junction-containing products in which only the top strands have been exchanged. This reaction requires the catalytic tyrosine residue of XerC but not that of XerD. Recombination at *psi* gives catenated circular resolution products. Strand exchange at *psi* is sequential. XerC catalyses the first (top) strand exchange to make a Holliday junction intermediate and XerD catalyses the second (bottom) strand exchange.

Keywords: *cer/psi*/recombinase/site-specific recombination/Xer

Introduction

Efficient segregation of circular replicons requires that they are in a monomeric state at cell division (Austin *et al.*, 1981; Summers and Sherratt, 1984). Multimerization by homologous recombination is an important cause of plasmid instability in multicopy plasmids. Xer-mediated site-specific recombination acts at sequences found in many naturally occurring multicopy plasmids (e.g. *cer* in ColE1 and *psi* in pSC101) to convert multimers to monomers, ensuring stable plasmid inheritance (Summers and Sherratt, 1984; Cornet *et al.*, 1994). Xer-mediated recombination also occurs at *dif*, a locus in the replication terminus region of the *Escherichia coli* chromosome. Recombination at *dif* is implicated in ensuring faithful segregation of the chromosome to daughter cells at cell division (Blakely *et al.*, 1991; Clerget, 1991; Kuempel *et al.*, 1991; Leslie and Sherratt, 1995).

Two chromosomally encoded recombinases, XerC and XerD, are required for all Xer-mediated recombination reactions (Colloms *et al.*, 1990; Blakely *et al.*, 1993). These two proteins are 37% identical at the amino-acid sequence level and have sequence similarity to the lambda integrase class of site-specific recombinases (Argos *et al.*, 1986; Abremski and Hoess, 1992). XerC and XerD both contain the four absolutely conserved residues (R, H, R and Y) of this family of site-specific recombinases. Three of these residues (R, H and R) are thought to be involved in activation of phosphodiester linkages in the cleavage and ligation reactions (Parsons *et al.*, 1988; Pan and Sadowski, 1992), while the conserved tyrosine (Y) is the catalytic nucleophile that becomes covalently linked to the DNA in the cleavage reaction (Pargellis *et al.*, 1988; Evans *et al.*, 1990).

The *dif* core site consists of an 11 bp XerC-binding site and an 11 bp XerD-binding site flanking a 6 bp central region. A 33 bp DNA fragment containing this *dif* core site is sufficient for Xer recombination *in vivo* (Blakely *et al.*, 1991; Leslie and Sherratt, 1995). Recombination at *dif* shows no resolution selectivity; intermolecular fusion is as efficient as intramolecular deletion at *dif* *in vivo* (Blakely *et al.*, 1991). In contrast, recombination at *cer* and *psi* is strongly biased towards intramolecular resolution, converting plasmid multimers to monomers and not vice versa. Recombination at *cer* requires 190 bp of accessory DNA sequences adjacent to a *dif*-like core recombination site with an 8 bp central region (Summers and Sherratt, 1988). Intramolecular recombination at *psi* requires at least 160 bp of accessory sequences adjacent to a core recombination site with a 6 bp central region (Cornet *et al.*, 1994; M.Burke and D.J.Sherratt, unpublished results).

In addition to XerC and XerD, recombination at *cer* requires two accessory proteins: PepA and ArgR (Stirling *et al.*, 1988a,b; 1989). These two proteins act in concert with the accessory sequences of *cer* to ensure that only intramolecular recombination occurs (Summers, 1989). ArgR is an L-arginine-dependent DNA-binding protein which binds to sequences upstream of the *E.coli* arginine biosynthetic operons (Glansdorff, 1987; Lim *et al.*, 1987). ArgR also binds to *cer* ~100 bp upstream of the point of strand exchange (Stirling *et al.*, 1988a). PepA is an aminoxo-peptidase whose role in Xer recombination has not been fully elucidated (Stirling *et al.*, 1989). However, the peptidase activity of PepA is not required for recombination at *cer* (McCulloch *et al.*, 1994b) and PepA has recently been identified as a DNA-binding protein involved in transcriptional regulation of the *carAB* operon of *E.coli* (Charlier *et al.*, 1995). Therefore, it seems likely that PepA plays a structural role, together with ArgR, in synapsis of *cer* sites prior to recombination.

Members of the lambda integrase family catalyse site-specific recombination in two steps: in the first step one

Fig. 1. Schematic representation of recombination substrates and products. Recombination between the two *cer* sites on pSDC115 produces a Holliday junction-containing molecule with two covalently closed supercoiled domains. Cleavage of one domain with *EcoRI* or the other domain with *Sall* produces a supercoiled α -structure and cleavage of both supercoiled domains (e.g. with *HincII*, or *EcoRI* and *Sall*) produces a χ -structure. RuvC treatment of α -structures can produce either a substrate length linear molecule or one nicked linear and one nicked circular product. Recombination between the two *psi* sites in pSDC134 produces two catenated circles of 3.05 kb and 1.65 kb. Cleavage with *EcoRI* or *XhoI* releases one supercoiled circle and one linear molecule from the catenane. Cleavage with *HindIII* releases two linear molecules from the catenane. Note that supercoiling and any catenation of the recombinant strands in the pSDC115 recombination product have been omitted for clarity. The exact structure of the catenane produced by recombination at *psi* will be discussed elsewhere (J.Bath, S.D.Colloms and D.J.Sherratt, unpublished data).

pair of strands is exchanged to form a Holliday junction, the second pair of strands is then exchanged to give recombinant product (Hsu and Landy, 1984; Hoess *et al.*, 1987; Nunes-Düby *et al.*, 1987; Kitts and Nash, 1988a,b). There is evidence that Xer-mediated recombination also goes through a Holliday junction intermediate. Xer-mediated strand exchange at *cer* *in vivo* leads to the accumulation of Holliday junction-containing molecules, which appear to be intermediates in the formation of recombinant product (McCulloch *et al.*, 1994a). Furthermore, synthetic Holliday junctions containing *cer*-related sequences are substrates for Xer-mediated strand exchange *in vitro*, although only XerC-catalysed top-strand exchange was observed with the substrates analysed (Arciszewska and Sherratt, 1995; Arciszewska *et al.*, 1995).

This report describes Xer-mediated recombination *in vitro*. Reactions were carried out on supercoiled substrates containing directly repeated recombination sites. Recombination at *cer* stops after XerC-mediated top-strand exchange, producing Holliday junction-containing products. Recombination at *psi* occurs by XerC-mediated top-strand exchange followed by XerD-mediated bottom-strand exchange, to produce a fully recombinant product via a Holliday junction intermediate.

Results

In vitro recombination of supercoiled substrates containing *cer* and *psi* sites

A negatively supercoiled plasmid (pSDC115, Figure 1) containing two directly repeated *cer* sites was incubated

with purified ArgR, PepA, XerC and XerD. The products were analysed by restriction enzyme digestion followed by agarose gel electrophoresis. Cleavage of the substrate (pSDC115) with *EcoRI* and *Sall* yields two linear fragments, each containing one *cer* site (Figure 1). Incubation of pSDC115 with recombination proteins for 1 h at 37°C converted ~30% of the substrate to a product (χ) which, when cleaved with *EcoRI* and *Sall*, had a mobility between linear and open circular forms of pSDC115 (Figure 2A, lane 1). This product was resistant to phenol extraction and protease treatment (data not shown). All four proteins, ArgR, PepA, XerC and XerD were necessary for the formation of this product (Figure 2A, lanes 2–5). We show below that this product has been formed by the exchange of one strand of each *cer* site in pSDC115, and therefore, contains a Holliday junction.

Recombination between *psi* sites *in vivo* requires PepA, XerC and XerD but not ArgR (Cornet *et al.*, 1994; data not shown). We incubated a supercoiled plasmid, containing two directly repeated *psi* sites (pSDC134, Figure 1) with PepA, XerC and XerD. The reaction products were analysed by *HindIII* restriction and agarose gel electrophoresis (Figure 2B). *HindIII* cuts pSDC134 twice, once on either side of each *psi* site (Figure 1). Two new bands were present (Figure 2B, lane 2) whose mobilities were as expected for *HindIII*-linearized pSDC134 resolution products. PepA, XerC and XerD were all required for recombination at *psi* (Figure 2B, lanes 3–5). The reaction did not require ArgR and the addition of ArgR had no effect (Figure 2B, lanes 1 and 2).

The *in vitro* reactions described above were greatly stimulated by the presence of either spermidine or a divalent cation such as Mg^{2+} (Figure 2A, lanes 1, 10 and 11, and B, lanes 1, 9 and 10). L-arginine was required for efficient recombination at *cer* (Figure 2A, lanes 1 and 9), presumably because it is required for the binding of ArgR to *cer* but had no effect on recombination at *psi*.

Recombination at *psi* was most efficient when PepA was used at a concentration 10- to 100-fold higher than that required for recombination at *cer*. When similar high concentrations of PepA were used in reactions at *cer*, recombination could occur in the absence of both ArgR and L-arginine (data not shown). It is possible that an additional 'ArgR-like' accessory protein is normally required for recombination at *psi* and that this requirement can be overcome by high concentrations of PepA.

Supercoiling is required for recombination reactions at both *cer* and *psi*. No recombination products were detected when singly nicked substrates were incubated with recombinase proteins in our standard conditions (data not shown). Fully relaxed covalently closed circular and linear substrates containing two recombination sites also failed to give any recombination products (data not shown).

Recombination *in vivo* at the *E. coli* chromosomal locus *dif* is dependent on XerC and XerD but is independent of ArgR and PepA. Supercoiled substrates containing two directly repeated *dif* sites were incubated with XerC and XerD, in the buffers used for *cer* and *psi* recombination, with and without PepA and ArgR. No recombination products were observed (data not shown). In other assays, we looked for intramolecular recombination between *dif* sites on linear or nicked DNA molecules and also for intermolecular recombination between *dif* sites on separate

Fig. 2. Protein requirements for recombination at *cer* and *psi*. (A) Supercoiled pSDC115, containing two directly repeated *cer* sites, was incubated for 1 h at 37°C with the proteins indicated (lanes 1–6). After phenol extraction and ethanol precipitation each reaction was cleaved with *Eco*RI and *Sa*II. The products were run on a 1.2% agarose gel. The samples in lanes 9–11 are identical to the sample in lane 1 except: lane 9, L-arginine was omitted; lane 10, spermidine was omitted; and lane 11, $MgCl_2$ was substituted for spermidine. Lane 7 contains *Eco*RI-linearized pSDC115 and lane 8 contains untreated pSDC115 as markers. (B) Supercoiled pSDC134, containing two directly repeated *psi* sites, was treated as above with the proteins indicated (lanes 1–6). Reactions were cleaved with *Hind*III prior to electrophoresis. The samples in lanes 9 and 10 are identical to the sample in lane 1 except: lane 9, spermidine was omitted and; lane 10, $MgCl_2$ was substituted for spermidine. Lane 7 contains *Eco*RI-linearized pSDC134 and lane 8 contains untreated pSDC134 as markers. Bands are indicated as follows: S, unrecombined substrate fragments (946 and 4020 bp for pSDC115, 983 and 3710 bp for pSDC134); lin, linearized substrate; oc, open circle substrate; χ , Holliday junction-containing recombination product; P, linearized pSDC134 recombination products (1647 bp and 3046 bp). Lane M contains 1 kb marker with linear fragments of the following sizes: 1.0, 1.6, 2.0, 3.0, 4.0, 5.0, 6.0, 7.0, 8.0, 9.0, 10.0 and 11.0 kb.

DNA molecules. Using these assays, we have so far been unable to detect any strand exchange between *dif* sites *in vitro*.

Recombination *in vitro* at *cer* yields Holliday junctions; recombination at *psi* yields catenanes

The mobility of the *Eco*RI–*Sa*II-cleaved *cer* reaction product *in vitro* (Figure 2A) suggested that it might be a χ -form, derived from a molecule containing a Holliday junction. If this is the case, cleavage with a restriction endonuclease which cuts once in pSDC115 would release an α -structure. Figure 3A (lanes 4 and 6) shows that cleavage of the pSDC115 reaction products with either *Eco*RI or *Sa*II gives a band corresponding to a supercoiled α -structure. The uncut pSDC115 reaction product has a mobility just higher than that of the supercoiled pSDC115 (Figure 3A, lane 2). These mobilities are the same as those of the Holliday junction-containing product of *in vivo* recombination on pSDC115 (McCulloch *et al.*, 1994a).

Further proof of the nature of the *cer* recombination product was obtained by treating the proposed α -structures with the Holliday junction-cleaving enzyme RuvC (Dunderdale *et al.*, 1991; Iwasaki *et al.*, 1991). RuvC acted on the putative α -structures to give the predicted linear and circular products (Figure 3A, lanes 11 and 12 and Figure 1). The α -structures are mostly converted by RuvC to nicked circle and small linear, rather than pSDC115-sized linear. It appears that the Holliday junction is held in a conformation in the supercoiled α -structure

in which RuvC preferentially cleaves the non-recombinant strands.

The uncleaved *cer* recombination product runs just faster than the supercoiled substrate (Figure 3A, lane 2), suggesting that this product retains most of its supercoiling. This conclusion is reinforced by the mobility of α -structures produced by cleavage of either domain of the product. These α -structures run faster than linear substrate, in the position expected if the closed domain is supercoiled. The product is fully religated, since any nick would allow the supercoiling to escape from the nicked domain. Therefore, the Holliday junction must have been produced by cleavage of one strand of each *cer* site, followed by swapping and religation of these strands, without any substantial loss of supercoiling between the cutting and religation steps of the reaction.

Recombination between two directly repeated sites on a circular molecule is expected to yield two circles, each containing one recombination site. As shown above, this is not the case for *cer*; the reaction stops after exchange of one pair of strands. In contrast, recombination at the *psi* sites on pSDC134 yielded catenated circular resolution products. The uncut pSDC134 recombination product ran distinctly faster than the supercoiled substrate and in the position expected for a supercoiled catenane (Figure 3B, lane 2). No uncatenated products were detected. *Hind*III cleavage of pSDC134 reaction products gave two bands of the correct size to be linearized product circles (Figure 3B, lane 10). Cleavage at unique restriction sites in

Fig. 3. Restriction analysis of recombination products. (A) pSDC115 was treated with ArgR, PepA, XerC and XerD (lanes 2, 4, 6, 8 and 10) or left untreated (lanes 1, 3, 5, 7 and 9). The reactions were run on a 1.2% agarose gel uncut (lanes 1 and 2) or cleaved with the restriction enzymes indicated (lanes 3–10). The reactions in lanes 11 and 12 were identical to those in lanes 4 and 6, respectively, except that they were treated with the Holliday junction-resolving enzyme RuvC after cleavage with restriction endonuclease. Lane 13 contains *Mlu*I cleaved pSDC115 as marker for linearized recombination products of 2.6 kb and 2.3 kb (*Mlu*I cleaves once within each *cer* site). The 2.6 kb replicative product of *in vivo* recombination on pSDC115, linearized with *Eco*RI (lane 14) or uncut (lane 15) was also loaded as marker. (B) pSDC134 was treated with PepA, XerC and XerD (lanes 2, 4, 6, 8 and 10) or left untreated (lanes 1, 3, 5, 7 and 9) and then run on a 1.2% agarose TAE gel, either uncut (lanes 1 and 2) or cleaved with the restriction enzymes indicated (lanes 3–10). The 3046 bp replicative product of *in vivo* recombined pSDC134, uncut (lane 11) or linearized with *Eco*RI (lane 12) were loaded as size markers. Bands are indicated as follows: ccc, lin and oc representing: supercoiled, linearized and open circle substrate respectively; HJ, supercoiled Holliday junction-containing product; α and χ , cleaved Holliday junction-containing products; 2621 ccc, 2621 lin and 2621 oc, supercoiled, linear and open circle forms of the 2621 bp (replicative) pSDC115 recombination product; 2345 lin and 2345 oc, linear and open circle forms of the 2345 bp pSDC115 recombination product; cat, supercoiled catenane recombination product; 3046 ccc and 3046 lin, supercoiled and linear forms of the 3046 bp pSDC134 recombination product; 1647 ccc and 1647 lin, supercoiled and linear forms of the 1647 bp pSDC134 recombination product; M, 1 kb marker as in Figure 2.

pSDC134 (Figure 3B, lanes 4 and 6) released one supercoiled circle and one linear molecule as expected for a catenane (Figure 1). Since cleavage of either circle in the catenane released the other circle in a supercoiled form, both circles in the catenane are covalently closed and supercoiled. Therefore, recombination at *psi* must occur via cleavage, exchange and religation of both strands of each *psi* site, without any substantial loss of supercoiling.

In all reaction conditions tested, Xer-mediated recombination at *cer* produced Holliday junctions and no fully recombinant product. In contrast, recombination at *psi* always yielded catenane as major product.

Recombination at both *cer* and *psi* *in vivo* preferentially converts multimers to monomers and not vice versa (Summers and Sherratt, 1984; Cornet *et al.*, 1994). However, *psi* can promote intermolecular recombination in some circumstances: a plasmid-borne *psi* core sequence promotes a low level of PepA-independent, XerC- and XerD-dependent multimerization (N.Ganguly and D.J.Sherratt, unpublished data), and plasmids carrying *psi* can integrate into the *E.coli* chromosome at *dif* (Cornet *et al.*, 1994). Despite this, recombination at both *cer* and *psi* is exclusively intramolecular in our *in vitro* system. Intramolecular deletion reactions take place efficiently but there is no intermolecular fusion (Figure 3A and B). In

experiments specifically designed to detect intermolecular reactions between *psi* sites *in vitro*, we have failed to detect any such reaction. Recombination between sites in inverted repeat takes place with low efficiency or not at all (data not shown).

The sequence of the recombination site core determines whether Holliday junction or catenane is produced

We wanted to find out what determines whether recombination produces fully recombinant catenane or stops after the first strand exchange to produce a Holliday junction. Therefore, we made two hybrid sites. One (*psi-cer*) contains the accessory sequences of *psi* and the core region of *cer*, the other (*cer-psi*) contains the accessory sequences of *cer* and the core region of *psi* (Figure 4). Two plasmids were constructed: pLN9 contains two directly repeated copies of *cer-psi*; pLN10 contains two directly repeated copies of *psi-cer*. Both of these plasmids recombine with reduced efficiency *in vivo* (data not shown). However, recombination *in vivo* at *cer-psi* required ArgR, PepA, XerC and XerD, whereas recombination at *psi-cer* required PepA, XerC and XerD but not ArgR (data not shown).

Efficient recombination *in vitro* at *cer-psi* (pLN9)

Fig. 4. The structure of *cer*, *psi* and the *cer-psi* hybrid sites. The *cer* site used in this study was the 284 bp *HpaII*–*TaqI* fragment from ColE1. The *psi* site used was the 360 bp *DdeI* fragment from pSC101. *cer* consists of an ~30 bp recombination core (C 8 D), containing an XerC-binding site and an XerD-binding site separated by an 8 bp central region, adjacent to accessory sequences of ~190 bp. The *cer* accessory sequences contain a site (ArgR) to which ArgR binds. *psi* consists of an ~30 bp core (C 6 D), containing an XerC-binding site and an XerD-binding site separated by a 6 bp central region, adjacent to accessory sequences of at least 160 bp. Only the recombination cores of *cer* and *psi* are similar. An *MluI* site (M) separates the recombination core from the accessory sequences of *cer*. An *MluI* site was created at the equivalent position within *psi* to give *psi-MluI*. The *MluI* sites in *cer* and *psi-MluI* were then used to make two hybrid sites: *cer-psi* and *psi-cer* as shown.

required ArgR, whereas recombination *in vitro* at *psi-cer* (pLN10) did not, consistent with the *in vivo* results (Figure 5). Therefore, the requirement for ArgR is determined by the accessory sequences; if these sequences come from *cer*, ArgR is required for efficient recombination; if they come from *psi*, ArgR is not required. The major product of recombination at *cer-psi* was catenane, although some Holliday junction-containing product was also formed (Figure 5). In contrast, recombination *in vitro* at *psi-cer* produced Holliday junction-containing product and not catenane (Figure 5). Therefore, the accessory sequences do not determine whether Holliday junction or catenane is produced. The accessory sequences of *cer* can participate in the formation of catenane (in *cer-psi*) or Holliday junction (in *cer*). Similarly the accessory sequences of *psi* can participate in the formation of Holliday junction (in *psi-cer*) and catenane (in *psi*). It is the core region of the recombination site which determines whether catenane or Holliday junction is produced. If the core sequence is that of *cer*, only one pair of strands is exchanged; if the core originates from *psi*, both pairs of strands can be exchanged.

The *cer* core contains an 8 bp central region between the XerC- and XerD-binding sites, whereas the *psi* core contains a 6 bp central region. To see if central region length is responsible for determining whether catenane or Holliday junction is produced, we looked at recombination at a variety of other sites. The results are summarized in Table I. Supercoiled plasmid substrates containing two directly repeated copies of *cer-6* or *cer-parB* (both with 6 bp central regions) or *nmr* (with a 7 bp central region) recombined *in vitro*, yielding Holliday junction-containing product and no catenane. Therefore, the length of the central region is not the sole determinant of whether one or two pairs of strands are exchanged.

***XerC* catalyses the first strand exchange, *XerD* catalyses the second strand exchange**

To investigate the role of XerC and XerD in the catalysis of recombination at *cer* and *psi*, we used recombinase

Fig. 5. Recombination at *cer-psi* and *psi-cer*. Recombination *in vitro* at *cer-psi* (A) and *psi-cer* (B) was analysed as follows. pLN9, containing two directly repeated copies of *cer-psi* and pLN10 containing two directly repeated copies of *psi-cer*, were incubated with the proteins indicated, cleaved with *NdeI* and *XhoI* and analysed on a 1.2% agarose gel. *NdeI* and *XhoI* cleave pLN9 and pLN10 to give two linear substrate fragments (S), of ~700 bp and 3300 bp, each containing one recombination site. Other bands are indicated as follows: χ , Holliday junction-containing product; P 1032 bp and 2970 bp linear fragments released by *XhoI*–*NdeI* cleavage from pLN9 catenane product.

proteins in which the active site tyrosine residue had been mutated to phenylalanine (XerCY275F and XerDY279F). These mutants bind to their respective binding sites with approximately normal affinity, but are unable to cleave DNA because they lack the tyrosine nucleophile responsible for this reaction. However, they allow a wild-type partner recombinase to carry out cleavage and strand-exchange reactions as normal (Arciszewska and Sherratt, 1995; N.Ganguly and D.J.Sherratt, unpublished data).

Replacing wild-type XerD with XerDY279F had no effect on the reaction at *cer in vitro*. (Figure 6A). In contrast, when XerDY279F was used in a *psi* recombination reaction, formation of catenanes was completely abolished and Holliday junction-containing products accumulated instead (Figure 6B). These products are covalently closed and supercoiled. Cleavage with unique restriction enzymes yielded supercoiled α -forms, which could be resolved by RuvC (data not shown).

A time course of recombination at *psi* and *cer*, using XerC and either XerD or XerDY279F, is shown in Figure 6C. All four reactions have approximately the same initial kinetics. At later time points, catenane, produced by XerC and XerD acting at *psi*, accumulates to much higher levels than Holliday junction-containing products produced by XerC and XerDY279F acting at *psi* or XerC and either XerD or XerDY279F acting at *cer*. One possible explanation for this observation is that the first strand exchange is reversible but the second strand exchange is not, so that Holliday junction can go back to substrate but catenane cannot. The small amounts of Holliday junction-containing product seen at early time points during the reaction at

Table 1. Effect of recombination core sequence on the type of product obtained

Site		XerC	Central region	XerD	Product
<i>cer</i>	<u>ACGCGTTTTAGC</u>	GGTGCCTACAA	TTAAGGGA	TTATGGTAAAT	Holliday junction
<i>cer-6</i>	<u>ACGCGTTTTAGC</u>	GGTGCCTACAA	GGGATG	TTATGGTAAAT	Holliday junction
<i>cer-parB</i>	<u>ACGCGTTTTAGC</u>	GGTACCGATAA	GGGATG	TTATGGTAAAT	Holliday junction
<i>nmr</i>	GCGCGTTTTGGC	GGTGCGCGTAA	TGAGACG	TTATGGTAAAT	Holliday junction
<i>psi</i>	TGGTGTTCGGGC	GGTGCGCGCAA	GATCCA	TTATGTTAAAC	Catenane

The recombination core sequences and the type of product obtained from *in vitro* recombination are tabulated for various sites. *cer-6* is the type II hybrid between *cer* and *parB* (Summers, 1989). *cer-parB* is a *cer* derivative in which the core region of *cer* has been replaced by that from *parB* (Hakkaart *et al.*, 1984). *nmr* is a recombination site from the naturally occurring plasmid pNTP16 (Cannon and Strike, 1992). The accessory sequences of these sites are not shown but note that *cer*, *cer-6* and *cer-parB* all have identical accessory sequences. The accessory sequences of *nmr* contain an ArgR-binding site and show ~50% identity to *cer* (Cannon and Strike, 1992). The *MluI* site of *cer* is shown underlined.

psi with XerC and XerD appear to be intermediates in the formation of catenane (Figure 6C).

When XerC was replaced by XerCY275F in *in vitro* recombination reactions at both *cer* and *psi*, recombination was completely abolished (Figure 6A and B). No Holliday junction-containing product or catenane was seen with either substrate.

These results show that Holliday junction formation at *cer* is catalysed by XerC. The active site tyrosine of XerD is not required for strand exchange at *cer* and even in the absence of the catalytic tyrosine of XerC, XerD cannot catalyse Holliday junction formation. Recombination at *psi* occurs by two ordered reciprocal strand-exchange reactions. XerC exchanges the first pair of strands, producing a Holliday junction, XerD exchanges the second pair of strands to give recombinant catenane. In the absence of the catalytic tyrosine of XerD the Holliday junctions, produced by XerC-catalysed strand exchange, accumulate. When XerC is lacking its catalytic tyrosine the first strand exchange cannot take place and no product is formed. The second, XerD-mediated strand exchange must be relatively fast because very little Holliday junction accumulates when wild-type recombinase proteins act at *psi*.

The effect of the XerCY275F and XerDY279F mutations on recombination *in vitro* are very similar to those seen *in vivo*. Recombination at both *cer* and *psi* was totally abolished *in vivo* by the XerCY275F mutation (Blakely *et al.*, 1993; data not shown). *In vivo* recombination at *cer* was just as efficient with XerDY279F as it was with wild-type XerD (data not shown), whereas recombination *in vivo* at *psi* was reduced but not abolished by the XerDY279F mutation (data not shown). The finding that XerDY279F is fully active at *cer in vivo* contradicts the findings of Blakely *et al.* (1993). However, the XerD mutant used by Blakely *et al.* also contained a second mutation (E184K, Blakely *et al.*, 1993) which we now know leads to reduced recombination *in vivo* (R.Baker and D.J.Sherratt, unpublished data).

Top strands of *cer* and *psi* are exchanged first

To ascertain which pair of strands is exchanged by XerC, Holliday junctions produced by wild-type proteins acting at *cer* or by wild-type XerC and XerDY279F acting at *psi* were cleaved to give χ forms and purified from an agarose gel. These χ forms were end-labelled with ^{32}P and analysed by gel electrophoresis in agarose TAE gels and in strand-separating agarose NaOH gels. The labelled χ forms produced four bands of the expected sizes on strand-separating gels (Figure 7). Labelled χ forms were

singly cleaved with a variety of restriction endonucleases and loaded on to agarose TAE and NaOH gels. Restriction cleavage produced a linear fragment labelled at one end and a χ labelled at three ends, both of which were seen on the TAE gel (not shown). On the strand-separating NaOH gel, restriction endonuclease-cleaved χ gave four bands, two of which are unchanged and two of which have been truncated by restriction endonuclease cleavage (Figure 7). The pattern of bands fits perfectly with that predicted for top-strand exchange and there is no sign of any bottom-strand exchange having taken place. Thus XerC catalyses top-strand exchange in recombination at both *cer* and *psi*. Since both top and bottom strands have been exchanged in the catenane product of recombination at *psi*, and XerD catalyses the second strand exchange in this reaction, it follows that XerD catalyses bottom-strand exchange at *psi*.

Discussion

The results presented here show that supercoiled plasmids containing directly repeated recombination sites are substrates for Xer-mediated recombination *in vitro*. The protein requirements for Xer recombination on supercoiled plasmid substrates are the same *in vitro* as they are *in vivo*. The accessory proteins ArgR and PepA, together with the accessory sequences, are implicated in ensuring that only intramolecular recombination occurs *in vivo* (Summers, 1989). This resolution selectivity is faithfully reproduced in our *in vitro* system. We hope that further *in vitro* studies will be informative about the exact mechanism that imposes topological selectivity on Xer recombination.

One possible mechanism to ensure resolution selectivity is the topological filter model, which has been proposed to explain topological selectivity in the reactions catalysed by Tn3/ $\gamma\delta$ resolvases and the DNA invertases (reviewed by Stark and Boocock, 1995). In these systems it is proposed that recombination can only occur after the formation of a synaptic complex with a fixed local geometry. One consequence of this type of mechanism is that the recombination products have a unique topology. The product of resolvase-mediated deletion is a singly interlinked (–2) catenane, the product of DNA invertase-mediated inversion is an unknotted circle. In contrast, lambda integrase and FLP, which do not utilize a topological filter mechanism, give products of mixed topologies. Therefore, it is interesting to note that Xer-mediated deletion at *psi* gives rise to a unique four-noded catenane (J.Bath, S.D.Colloms and D.J.Sherratt,

Fig. 6. Recombination at *cer* and *psi* with mutants of XerC and XerD. (A) pSDC115 was incubated with ArgR, PepA, and wild-type or mutant XerC and XerD as indicated. Reactions were cleaved with *SalI* and *EcoRI* and analysed on a 1.2% agarose gel. (B) pSDC134 was incubated with PepA, and wild-type or mutant XerC and XerD as indicated. Reactions were cleaved with *HindIII* and run on a 1.2% agarose gel. Bands are indicated as follows: χ , Holliday junction-containing products; S, substrate bands; P, linearized pSDC134 recombination products. (C) Kinetics of recombination at *cer* and *psi* with XerD⁺ and XerDY279F. pSDC115 (*cer*) and pSDC134 (*psi*) were incubated with ArgR (when appropriate), PepA, XerC and either XerD (open symbols) or XerDY275F (filled symbols). Samples were taken at 1, 4, 15 and 60 min, stopped by phenol extraction, ethanol precipitated and cleaved with either *EcoRI* and *SalI* (pSDC115) or *HindIII* (pSDC134) before electrophoresis on a 1.2% agarose gel. For each time point, the amount of product (cleaved catenane and χ for pSDC134 with XerD⁺, χ for all others) was determined as a percentage of the total amount of DNA, using a Molecular Dynamics FluorImager 575. A small amount of χ was seen at the early time points when pSDC134 was recombined with XerD⁺.

Fig. 7. Holliday junctions produced by Xer recombination are the products of top-strand exchange. Holliday junctions produced by recombination at *cer* (A) or at *psi* in the presence of XerDY279F (B) could be the products of either top- or bottom-strand exchange or a mixture of both as diagrammed. (A) χ -form DNA was made by treating pSDC115 with ArgR, PepA, XerC and XerD followed by cleavage with *EcoRI* and *SalI*. χ was purified from an agarose gel and 5' end-labelled with ³²P. Labelled χ was then run on a strand-separating NaOH agarose gel uncut or cleaved with *EcoRV*, *HincII*, *NruI* or *SalI*. (B) χ -form DNA was made by treating pSDC134 with PepA, XerC and XerDY279F, followed by cleavage with *HindIII*. χ -form DNA was purified from an agarose gel and 3' end-labelled with ³²P. Labelled χ was then run on a strand-separating NaOH agarose gel uncut or cleaved with *Clal*, *EcoRI*, *XhoI* or *NdeI*. Predicted sizes of labelled single strands for the uncut χ s (top or bottom strands exchanged) are: pSDC115: 4020, 2621, 2345 and 946 nucleotides; pSDC134: 3710, 3046, 1647 and 983 nucleotides. Top- and bottom-strand exchange are expected to give different patterns only after restriction. For example *NruI* cleavage of pSDC115 χ should give single strands of: 323, 946, 2621 and either 2026 (bottom-strand exchange) or 3701 nucleotides (top-strand exchange); *NdeI* cleavage of pSDC134 χ should give single strands of: 219, 983, 1647 and either 2829 (bottom-strand exchange) or 3493 nucleotides (top-strand exchange). Filled triangles represent the XerD-binding sites, pointing away from the accessory sequences. Small filled circles represent ³²P 5' end-label (A) or ³²P 3' end-label (B).

unpublished data). Therefore, it seems possible that the resolution selectivity displayed during recombination at *cer* and *psi* may employ a similar topological filter to that

used by the resolvase/DNA invertase class of site-specific recombinases. This topological filter could involve interwrapping of *cer* or *psi* accessory sequences around the accessory proteins PepA and ArgR to trap a specific number of nodes. If this is indeed the case, it would be the first example of such a topological filter being used by a member of the lambda integrase family of site-specific recombinases.

Recombination at *cer* gives rise to Holliday junction-containing product in which only one pair of strands has been exchanged. Recombination at *psi* gives fully recombinant product via a Holliday junction intermediate; Holliday junctions are seen at early time points during the reaction and accumulate if the second strand exchange is blocked. Thus, Xer-mediated recombination proceeds by sequential exchange of two pairs of strands, as is thought to be the case for reactions catalysed by all other members of the lambda integrase family of recombinases (Hsu and Landy, 1984; Hoess *et al.*, 1987; Nunes-Düby *et al.*, 1987; Kitts and Nash, 1988a,b; Meyer-Leon *et al.*, 1988, 1990). Earlier work also pointed to the involvement of Holliday junctions in the Xer recombination system: Holliday junctions appear as intermediates in recombination at *cer in vivo* (McCulloch *et al.*, 1994a); and synthetic Holliday junctions are substrates for Xer-mediated strand exchange *in vitro* (Arciszewska and Sherratt, 1995; Arciszewska *et al.*, 1995). In contrast, recombination catalysed by the resolvase/DNA invertase family occurs by a concerted four-strand cleavage and religation mechanism (reviewed in Stark *et al.*, 1992).

The catalytic tyrosine of XerD is required for bottom-strand exchange at *psi*. This is the first conclusive proof that XerD can catalyse strand exchange reactions. Only XerC-mediated top-strand exchange was observed when Holliday junctions were used as substrates (Arciszewska and Sherratt, 1995; Arciszewska *et al.*, 1995), although small amounts of XerD-mediated cleavage have since been seen on linear 'suicide' substrates containing a nick or gap between the recombinase-binding sites (G.Blakely and D.J.Sherratt, unpublished data). The catalytic tyrosine of XerD is not required for top-strand exchange at either *psi* or *cer* but XerD must be present. This presumably reflects co-operative interactions between XerC and XerD at the DNA-binding step or at other steps in the strand exchange reaction.

Recombination at *psi* proceeds with a defined order of strand exchanges, top-strand exchange must occur before bottom-strand exchange can take place. Recombination by lambda integrase also occurs with a defined order of strand exchanges (Kitts and Nash, 1988b). In the case of lambda integrase, it is the orientation of the *attP* arms (P and P') with respect to the recombination core that defines the order of strand exchange. The asymmetry which results in ordered strand exchange at *cer* and *psi* may reside in: (i) the disposition of the accessory sequences to one side of the recombination core, (ii) the asymmetry of the core with the XerC-binding site on one side and the XerD-binding site on the other or (iii) asymmetry in the 6–8 bp central region between the XerC and XerD-binding sites. The first possibility could involve a synaptic complex, formed by interwrapping of accessory sequences around the accessory proteins, aligning the recombination cores in a spatial configuration in which only XerC-catalysed

top-strand exchange can take place. Top-strand exchange, isomerization and branch migration could then allow XerD-catalysed bottom-strand exchange.

The major difference between reactions at *cer* and *psi in vitro* is that *cer* produces Holliday junction whereas *psi* produces catenane. Is this also the case *in vivo*? During a time course of recombination at *cer in vivo*, Holliday junctions appear early in the reaction and persist for quite some time (McCulloch *et al.*, 1994a). No such Holliday junctions were seen when a similar *in vivo* time course was carried out with *psi* (R.Baker and D.J.Sherratt, unpublished data). Product circles are produced by recombination at *cer in vivo* and eventually predominate (McCulloch *et al.*, 1994a). However, these product circles are formed just as efficiently in the presence of XerDY279F as in the presence of XerD⁺ (R.Baker and D.J.Sherratt, unpublished data). In contrast, recombination at *psi in vivo* is much reduced in the presence of XerDY279F. It seems that recombination at *cer in vivo* occurs by XerC-mediated Holliday junction formation, followed by some non-Xer process to give product circles, whereas recombination at *psi in vivo* goes by XerC-mediated Holliday junction formation followed by XerD-mediated Holliday junction resolution. We do not know what *in vivo* process is responsible for resolving the Holliday junctions formed at *cer*, but it does not require RuvC (McCulloch *et al.*, 1994a). The biological significance of this difference between *cer* and *psi* is not clear.

We have shown that the sequence of the recombination site core is important in determining whether the reaction stops at Holliday junction or carries on to give the second strand exchange. The *psi* core is the only sequence identified so far which allows this second strand exchange reaction to take place. The exact sequence determinants which allow the *psi* core to undergo the second strand exchange are not yet known, but the length of the central region between the XerC- and XerD-binding sites is not the sole determinant, so some other sequence difference must play a role. Holliday junction isomerization, XerD-mediated bottom-strand cleavage, or some other step could be blocked by the non-*psi* core sequences.

Using the assays described, we have been unable to detect recombination between *dif* sites *in vitro*. This may reflect some type of *in vivo* control imposed on recombination at *dif*. Xer recombination at plasmid sites requires accessory proteins and sequences, which ensure that only intramolecular resolution takes place. Perhaps there is some other genetic process, absent from our *in vitro* system which ensures that recombination at *dif* helps rather than hinders chromosome segregation.

Materials and methods

Bacterial strains, plasmids and growth conditions

All strains are derivatives of *E.coli* K-12 AB1157 (Bachmann, 1972). DS941 is AB1157 *recF lac^R lacΔM15*. DS984 is DS941 *xerCY17*. DS9029 is DS941 *xerCY17 xerD::fol*. pKS493 contains *cer* as a 284 bp *HpaII*-*TaqI* fragment from ColE1 inserted into pUC18 (Stirling *et al.*, 1988b). pSDC115 contains two directly repeated copies of *cer* in pBR322 (McCulloch *et al.*, 1994a). pSDC133 was constructed by inserting *psi* as a 360 bp, Klenow-filled *DdeI* fragment of pSC101 (coordinates 6502–6861) into the *SmaI* site of pUC18. The *psi* reporter plasmid pSDC134 was constructed by ligating the 0.6 kb *EcoRI*-*NdeI* fragment of pSDC133 together with the 2.8 kb *NdeI*-*HincII* fragment of pSDC133 and the 1.3 kb *Tn903 Kan^r* gene as a *HincII* fragment from pUC4K (Pharmacia).

An *MluI* site was introduced into the *psi* site of pSDC133 using the Promega Altered Sites kit and the oligonucleotide 5'-CGCACCGCCCCG-AACGCGTCTCGCCACAAAAAC-3' creating pLN5. Exchange of *NdeI*-*MluI* fragments between pLN5 and pKS493 gave pLN6 (containing *cer-psi*) and pLN7 (containing *psi-cer*). pLN9 and pLN10, containing two directly repeated copies of *cer-psi* and *psi-cer* respectively, were constructed from pLN6 and pLN7 by ligating the 0.6 kb *NdeI*-*EcoRI* fragment and the 2.8 kb *NdeI*-*HindIII* fragment from the parental plasmid together with a 0.7 kb *EcoRI*-*HindIII* fragment from pUC4k. pSDC2000 contains *cer-6* (also known as the type II *cer-parB* hybrid) complete with *cer* accessory sequences in pUC18 and is equivalent to pKS2000 (Guhathakurta and Summers, 1995). pJEN42 contains *cer-parB* (J.Roberts, unpublished data), a *cer* derivative in which the core of *cer* has been replaced by that of CloDF13 *parB* (Hakkaart *et al.*, 1984) in pUC18. pUCnmr contains a 0.48 kb *HincII*-*DraI* fragment of NTP16 (coordinates 1960–2443; Cannon and Strike, 1992) cloned into the *SmaI* site of pUC18. pSDC160, pSDC162 and pSDC163 were constructed in a similar fashion to pLN9 and pLN10 from plasmids pJEN42, pSDC2000 and pUCnmr containing *cer-parB*, *cer-6* and *nmr* respectively. pAS100 and pSDC141 carry *xerC* and *xerCY275F* in the expression vector pTrec99a (Pharmacia, A.Spiers, unpublished data). pRM130 and pLA137 carry *xerD* and *xerDY279F* in pUC19 (Blakely *et al.*, 1993; Arciszewska and Sherratt, 1995). Bacteria were grown in LB medium (Sambrook *et al.*, 1989), supplemented with antibiotics as appropriate. Supercoiled plasmid substrates for *in vitro* recombination were prepared from DS984 by large scale alkaline lysis followed by CsCl-ethidium bromide density gradient centrifugation.

Proteins

ArgR was purified as described by Burke *et al.* (1994). PepA was purified essentially as described in McCulloch *et al.* (1994b). RuvC was the kind gift of Steve West. XerC and XerCY275F were prepared as follows: an overnight culture of DS9029 carrying either pAS100 or pSDC141 was diluted 50-fold into 500 ml of fresh LB broth supplemented with 100 µg/ml ampicillin and grown at 37°C until it reached $A_{600} = 0.4$. Protein expression was induced by the addition of IPTG and growth was continued for a further 3 h at 37°C. The cells were harvested by centrifugation, and stored overnight at –70°C. The cell pellet was resuspended in 6 ml of buffer A (50 mM NaCl, 50 mM Tris-HCl pH 8.0 and 1 mM EDTA) supplemented with 0.1% Nonidet P40 and the cells were disrupted by sonication. The majority of XerC is insoluble and was recovered by centrifugation at 12 000 g for 15 min. The pellet was washed twice in buffer A and then resuspended in buffer A + 8 M urea. Insoluble material was removed by centrifugation at 12 000 g for 15 min and the supernatant was dialysed in three steps, each of 1 h at 4°C: first, in buffer A + 4 M urea, then in buffer A + 2 M urea and finally in buffer A + 1 M urea. The dialysate was centrifuged again and applied to a MonoS column (Pharmacia) previously equilibrated to buffer A + 1 M urea. XerC was eluted with a 0.05–1.0 M NaCl linear gradient in buffer A + 1 M urea. Peak fractions (eluting at ~0.5 M NaCl) were made up to 50% glycerol and dialysed against 50% glycerol, 1 M NaCl, 50 mM Tris-HCl pH 8.0 and 1 mM EDTA overnight at 4°C and stored at –20°C. The resulting fractions contain ~0.2 mg/ml XerC and were estimated to be 75% XerC by Coomassie stained SDS-PAGE. The major contaminant appears to be a proteolytic fragment of XerC.

XerD and XerDY279F were purified from DS9029 carrying either pRM130 or pLA137. Protein was expressed and cells were pelleted as described for XerC. The cell pellet was re-suspended in 5 ml of lysis buffer (100 mM KCl, 50 mM Tris-HCl pH 8.0 and 1 mM EDTA), lysozyme (0.5 mg/ml) was added and the suspension was left on ice for 20 min. The cells were lysed by sonication and the lysate was cleared by centrifugation at 27 000 g. The cleared lysate was mixed with 8 ml of a 50% slurry of Ni-NTA resin (Qiagen), equilibrated in lysis buffer and incubated for 30 min at 4°C with gentle mixing. The slurry was poured into a column and then washed with 30 ml of wash buffer (20 mM Na-acetate pH 5.0, 10 mM NaCl and 1 mM EDTA) at 4°C. XerD was eluted with 15 ml of elution buffer (20 mM Na-acetate, 10 mM NaCl, 1 mM EDTA and 250 mM imidazole pH 8.4). Note that XerD binds to Ni-NTA although it has no His-tag. Peak fractions were pooled, dialysed against buffer B (50 mM Tris-HCl pH 8.0, 50 mM NaCl and 1 mM EDTA) and loaded onto a MonoS column (Pharmacia) previously equilibrated to buffer B. XerD was eluted with a 0.05–1.0 M NaCl gradient with buffer B. Peak fractions (eluting at about 0.45 M NaCl) were diluted with an equal volume of glycerol and stored at –20°C. The resulting XerD preparation contains ~0.4 mg/ml XerD and is >80% XerD. The XerDY279F preparation contains ~0.2 mg/ml and is ~60% XerD.

In vitro recombination assays

A typical recombination reaction contained ~300 ng of supercoiled plasmid substrate in a volume of 20 µl. Reactions on substrates containing *cer* or other substrates with *cer* or *cer*-like accessory sequences, were set up in '*cer* buffer': 50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1.25 mM EDTA, 5 mM spermidine, 1 mM L-arginine, 10% glycerol and 25 µg/ml BSA. Reactions on substrates containing *psi* or *psi-cer* were set up in '*psi* buffer' which is identical to '*cer* buffer' except that L-arginine was omitted and 25 mM KCl was substituted for 50 mM NaCl. Reactions at *cer* and *cer*-like sites were started by the addition of 0.1 µl ArgR, 0.01–0.1 µl PepA, 1 µl XerC and 0.5 µl XerD, in that order. Reactions at *psi* and *psi-cer* were started by the addition of 1 µl PepA, 1 µl XerC and 0.5 µl XerD. Estimated final concentrations are as follows: substrate DNA, 6 nM; ArgR, 30 nM hexamer; PepA 3–30 nM or 300 nM hexamer; XerC, 300 nM monomer; and XerD, 300 nM monomer. Reactions were normally carried out for 1 h at 37°C and stopped by extraction once with phenol, once with phenol/chloroform and once with chloroform. DNA was ethanol precipitated, resuspended in TE or H₂O, treated with restriction enzymes as appropriate and analysed by electrophoresis on 1.2% agarose TAE gels. RuvC cleavage of Holliday junctions was carried out by adding 2 µl 10× RuvC cleavage buffer (500 mM Tris-HCl pH 8.0, 10 mM DTT, 100 mM MgCl₂ and 1 mg/ml BSA) and 0.1 µl RuvC to a 20 µl completed restriction digest. RuvC reactions were incubated at 37°C for 30 min, extracted once with phenol, once with phenol-chloroform, once with chloroform and loaded directly onto agarose gels. DNA was visualized by 254 nm UV illumination after staining with ethidium bromide and photographed onto Ilford HP5 film. For quantitation, gels were stained with ethidium bromide or SYBR green (molecular probes) and analysed using a Molecular Dynamics FluorImager 575.

Analysis of χ -DNA by strand-separating gel electrophoresis

A standard *in vitro* recombination reaction was carried out on pSDC115 as described above. After cleavage with *EcoRI* and *Sall* and treatment with alkaline phosphatase, χ -form DNA was purified from a 'Seaplaque' (FMC) low melting point agarose gel by phenol extraction and ethanol precipitation. Purified pSDC115 χ -form DNA was 5' end-labelled with γ -³²P-labelled ATP (Amersham) and T4 polynucleotide kinase. pSDC134 χ -form DNA was isolated as above except that XerDY279F was used in the recombination reaction and *HindIII* was used instead of *EcoRI* and *Sall*. Purified pSDC134 χ -form DNA was 3' end-labelled with α -³²P-labelled dATP and the Klenow fragment of DNA polymerase I in the presence of unlabelled dCTP, dGTP and dTTP. Labelled χ -form DNA, uncut and cut with appropriate restriction enzymes, was analysed by electrophoresis on 1% agarose, TAE gels and on 1% agarose, 50 mM NaOH, 1 mM EDTA strand-separating gels, as described by McCulloch *et al.* (1994a). The DNA was transferred to Hybond-N (Amersham) by capillary blotting (following the procedure recommended by Amersham) and detected by autoradiography.

Acknowledgements

We thank Steve West for the gift of RuvC. Amir Merican for supplying ArgR and Andrew Spiers for supplying all of the wild-type XerD used in this study. Many thanks go to Jennifer Roberts for constructing pJEN42 and to Peter Strike and George Szatmari for supplying pUCnmr. Thanks also go to Marshall Stark for his constructive criticism of the manuscript. This work was supported by the BBSRC, the MRC, the Nuffield Foundation and the Wellcome Trust.

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Received on October 10, 1995; revised on November 8, 1995