FtsK-dependent and -independent pathways of Xer site-specific recombination

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Homologous recombination between circular chromosomes generates dimers that cannot be segregated at cell division. *Escherichia coli* Xer site-specific recombination converts chromosomal and plasmid dimers to monomers. Two recombinases, XerC and XerD, act at the *E. coli* chromosomal recombination site, *dif*, and at related sites in plasmids. We demonstrate that Xer recombination at plasmid *dif* sites occurs efficiently only when FtsK is present and under conditions that allow chromosomal dimer formation, whereas recombination at the plasmid sites *cer* and *psi* is independent of these factors. We propose that the chromosome dimer- and FtsK-dependent process that activates Xer recombination at plasmid *dif* also activates Xer recombination at chromosomal *dif*. The defects in chromosome segregation that result from mutation of the FtsK C-terminus are attributable to the failure of Xer recombination to resolve chromosome dimers to monomers. Conditions that lead to FtsK-independent Xer recombination support the hypothesis that FtsK acts on Holliday junction Xer recombination intermediates.

Keywords: *dif*/FtsK/site-specific recombination/Xer

Introduction

Genetic information must not only be accurately replicated at each cell generation, but be faithfully segregated to daughter cells at cell division. In organisms containing circular chromosomes, their segregation at cell division is compromised because homologous recombination between circular chromosomes can generate dimers which must be converted to monomers if intact monomeric chromosomes are to be segregated to daughter cells (McClintock, 1932). Catenation linking monomeric chromosomes must also be removed by topoisomerases prior to chromosome segregation. Most homologous recombination in *Escherichia coli* is thought to occur between newly replicated homologous DNA segments during RecA-dependent recombinational DNA repair of stalled or broken replication forks (reviewed in Cox, 1998). *Escherichia coli* Xer site-specific recombination acts to convert circular DNA dimers to monomers (Sherratt *et al.*, 1995). Most other bacteria appear to encode a related recombination system (Esposito and Scoocca, 1997; Nunes-Düby *et al.*, 1998).

In *E. coli*, two tyrosine-family site-specific recombinases, XerC and XerD, bind co-operatively at a specific chromosomal site, *dif*, located in the replication terminus region (Blakely *et al.*, 1991, 1993; Kuempel *et al.*, 1991), and at related sites present in natural multicopy plasmids (Summers and Sherratt, 1984; Sherratt, 1993; Cornet *et al.*, 1996). XerC initiates recombination by catalysing the first pair of strand exchanges to form a Holliday junction (HJ) intermediate, which is acted on by XerD in reactions that lead to complete recombinant products (Colloms *et al.*, 1996, 1997; Neilson *et al.*, 1999). Deletion of *dif* or mutations in the *xerC* or *xerD* genes results in aberrant chromosome segregation (Blakely *et al.*, 1991; Kuempel *et al.*, 1991). The location of the *dif* site in the terminus region of the chromosome is important for its role in chromosome segregation, since translocation of *dif* to any location >20–30 kb from its natural position results in a *Dif*[−] phenotype (Leslie and Sherratt, 1995; Tecklenburg *et al.*, 1995; Cornet *et al.*, 1996; Kuempel *et al.*, 1996). The minimal *dif* site required for activity is contained within a 33 base pair (bp) core sequence consisting of 11 bp binding sites for XerC and XerD flanking a 6 bp central region (Leslie and Sherratt, 1995; Tecklenburg *et al.*, 1995).

The Xer recombination system has also been recruited by a number of multicopy plasmids, which include ColE1 and its relatives (Sherratt, 1993), and pSC101 (Cornet *et al.*, 1996), to resolve multimers prior to their segregation to daughter cells. Recombination at the natural plasmid sites *cer* (ColE1) and *psi* (pSC101) is preferentially intramolecular, ensuring that multimers are resolved to monomers. Both *cer* and *psi* also possess, in addition to their core sites which are homologous to the *dif* core site, ~200 bp of accessory sequences. Host-encoded proteins bind to these accessory sequences to give a nucleoprotein complex that forms and functions effectively only on directly repeated sites contained within a molecule (Blakely and Sherratt, 1996; Colloms *et al.*, 1996, 1997). In contrast, *dif* contains no accessory sequences, and undergoes inter- and intramolecular recombination (Blakely *et al.*, 1991; Kuempel *et al.*, 1991; Cornet *et al.*, 1994; Leslie and Sherratt, 1995). Although synthetic *dif*-containing HJs are excellent substrates for Xer recombination (Arciszewska *et al.*, 1997), supercoiled molecules containing *dif* are unable to recombine *in vitro* with *E. coli* XerC and XerD (Colloms *et al.*, 1996). This may reflect the *in vivo* control of Xer recombination at *dif* that is addressed in this report.

Measurements of *dif* recombination *in situ* within the chromosome have shown that recombination at *dif* is dependent on the initiation of cell division (Steiner and Kuempel, 1998a), appears to be proportional to the level of homologous recombination that the cells can support (Steiner and Kuempel, 1998b), and requires the C-terminal

domain of FtsK (Steiner *et al.*, 1999). FtsK is a 1329-amino-acid protein that appears to have three domains. An ~180-amino-acid N-terminal transmembrane domain (domain I) localizes the protein to the septum in an FtsZ-dependent manner and is essential for cell division (Draper *et al.*, 1998; Wang and Lutkenhaus, 1998; Yu *et al.*, 1998b). The ~480-amino-acid C-terminal cytoplasmic domain (domain III) is implicated in chromosome segregation and contains a presumptive ATP-binding site (Liu *et al.*, 1998; Yu *et al.*, 1998a). The C- and N-terminal domains are separated by a region of ~700 amino acids (domain II) which is proline- and glutamine-rich (Begg *et al.*, 1995). FtsK exhibits homology with the *Bacillus subtilis* SpoIIIE protein, which is implicated in the transport of chromosomal DNA into the prespore, and proteins implicated in plasmid conjugal transfer in Gram-positive bacteria (Wu *et al.*, 1995). Furthermore, genomic DNA sequences that encode peptide sequences homologous to domains I and III are present in many bacterial species.

Here we demonstrate that Xer recombination at plasmid-borne *dif*, and between plasmid *dif* and chromosomal *dif*, requires FtsK containing a functional C-terminus and conditions which allow the formation of chromosomal dimers. Furthermore, we demonstrate that Xer recombination at the natural plasmid sites *cer* and *psi* is independent of these factors. Our results are consistent with the view that a chromosomal dimer-dependent and FtsK-dependent process leads to activation of Xer recombination at *dif*. Our results also demonstrate that the defects in chromosome segregation that result from mutation of the FtsK C-terminus are attributable to the failure of Xer recombination at *dif* to resolve chromosome dimers.

Results

Xer recombination between directly repeated plasmid-borne dif sites is dependent on a functional chromosomal homologous recombination system but not on the ability to induce the SOS response

Steiner and Kuempel (1998b) have shown that the frequency of Xer recombination at chromosomal *dif* is proportional to either the cell's ability to support homologous recombination, or the expected level of homologous recombination (Steiner and Kuempel, 1998b). In order to understand more about how Xer recombination at *dif* relates to chromosome metabolism and the cell cycle, we compared recombination between *dif* sites contained within a multicopy plasmid in a number of bacterial strains that vary in their ability to mediate homologous recombination and in their SOS response.

Conversion of a plasmid containing two directly repeated *dif* sites (pSDC124) to a derivative containing a single *dif* site is dependent on the presence of XerC and XerD, and can occur by either intramolecular recombination or a combination of inter- and intramolecular recombination. In the latter case, a mixture of equal and unequal reciprocal exchanges generates plasmids containing a single *dif* site and three *dif* sites, respectively. A comparison of the levels of plasmid substrate (S) to resolution product (RP) and Xer-dependent multimers for the various strains is shown in Figure 1A. The RecA⁻ strain contains fewer Xer recombination products than the

Fig. 1. (A and B) Xer recombination between plasmid-borne *dif* sites is reduced in a RecA⁻ strain. Strains are: AB1157 (WT), DS941 (*recF*), GR19 (*recA*), DS931 (*lexA3*), GR35 (*dif*⁻) and DS8029 (*xerC2*). S, pSDC124 plasmid substrate; RP, resolved product plasmid. (A) Analysis of supercoiled DNA. Arrows indicate DNA species attributable to Xer recombination, derived by comparison of AB1157 (WT) and its *xerC* derivative (DS8029). (B) Analysis of the plasmid DNA in (A) after digestion with *NdeI*, which cleaves the plasmid backbone once. The bands containing three and four *dif* sites can arise by Xer-mediated reinsertion of the small non-replicating circle containing a single *dif* site into a plasmid containing two or three *dif* sites, or by unequal intermolecular Xer recombination. Each of the apparent product bands may be derived from monomeric or multimeric plasmids. For reasons we do not understand the fragment containing three *dif* sites is more constant between strains than the fragment containing a single *dif* site. (C) Recombination at *cer* and *psi* is RecA-independent. Analysis of supercoiled DNA isolated from AB1157 (Rec⁺) and GR19 (Rec⁻). Symbols are as in (A).

RecA⁺ control. In contrast, the *lexA3* strain, which is not able to induce the SOS response, as it has an uncleavable LexA repressor, gave a similar level of products as the wild-type control. In order to confirm these observations, and remove any ambiguity from the fact that multimerization of pSDC124 or its resolution product can occur by both Xer and homologous recombination, the level of Xer recombination was also scored by analysing the products after restriction endonuclease digestion, where only DNA molecules that are necessarily dependent on Xer recombination for their formation are assayed (Figure 1B). This underestimates the total extent of Xer recombination, since the products that can be generated by both Xer and homologous recombination are not assayed (compare the apparent levels of Xer recombination in Figure 1A and B). Nevertheless, both assays give the same conclusion: in the absence of RecA protein the level of Xer recombinant

Fig. 2. Xer recombination at plasmid-borne *dif* as a function of time after induction of XerC synthesis. Glucose-grown *RecA*⁺ (RM40) and *RecA*⁻ (DW1) derivatives of a strain in which chromosomal XerC is expressed from the wild-type *plac* promoter were transformed with plasmid pSDC124. (A) Plasmid DNA, isolated at the indicated times after IPTG induction, was analysed by electrophoresis after *NdeI* digestion. During the 180 min of IPTG induction the amount of total plasmid DNA increased 7-fold and 6-fold in the *RecA*⁺ and *RecA*⁻ strains respectively. S, pSDC124 plasmid substrate; RP, resolved product. (B) Relative amounts of recombinant product generated by Xer recombination between pSDC124 *dif* sites in RM40 and DW1, quantified from the gel in (A). The percentage of recombinant product was measured as the proportion of total plasmid DNA present as resolved product (RP) plus the DNA fragment containing three *dif* sites. Circles represent RM40 (*RecA*⁺), and squares represent DW1 (*RecA*⁻).

product is reduced, whereas the level of product in a strain containing the *lexA3* allele is similar to that of the *RecA*⁺ control. These observations indicate that the reduced level of Xer recombinant products in the *RecA*⁻ strain is a consequence of the lack of homologous recombination rather than a failure to induce the SOS response.

Because the relative amounts of the different plasmid species in the above experiment are a consequence of both recombination and replication of the various plasmid forms during a period of ~40 generations, we reduced any effects of replication by analysing Xer recombination in cells over a 180 min period after expression of a chromosomal *xerC* gene from the wild-type *plac* promoter (McCulloch *et al.*, 1994). In glucose-grown *RecA*⁺ or *RecA*⁻ cells, there was little detectable XerC expression as judged by the level of Xer recombination between *dif* sites on pSDC124. Isopropyl-β-D-galactopyranoside (IPTG) induction led to a steady increase in the level of Xer recombinant product in the *RecA*⁺ strain over 180 min (Figure 2A), by which time ~17% of pSDC124 had been converted to resolution product (Figure 2B). In contrast, in the *RecA*⁻ strain, the initial rate of appearance of recombinant product was considerably slower and by 180 min was only ~5% of total plasmid.

We conclude that there is a clear difference between the frequency of Xer recombination at plasmid *dif* in

RecA⁻ and *RecA*⁺ strains, although Xer recombination at plasmid-borne *dif* sites is not totally *RecA*-dependent, whereas Xer recombination at chromosomal *dif* was not detected in a *RecA*⁻ strain (Steiner and Kuempel, 1998a,b). In contrast, recombination at *cer* and *psi* is *RecA*-independent (Figure 1C).

The observation that a *RecF*⁻ strain shows similar levels of Xer recombinant product to the *RecF*⁺ parent (Figure 1) demonstrates that the requirement for *RecA* function is not a consequence of homologous recombination being required on the plasmid containing *dif*, since pUC-related plasmids undergo little homologous recombination in *RecF*⁻ mutants (Summers and Sherratt, 1984; Kolodner *et al.*, 1985), whereas these strains support substantial levels of chromosomal homologous recombination (Steiner and Kuempel, 1998b). The simplest interpretation of these data is that chromosomal homologous recombination, but not plasmid homologous recombination, is required for efficient Xer recombination at plasmid-borne *dif*.

In order to test if *RecA*-dependent Xer recombination at plasmid *dif* requires the presence of chromosomal *dif*, we compared Xer recombination at *dif* in pSDC124 in strains containing *dif*⁺ and *dif*⁻ chromosomes. In the *dif*⁻ strain, the levels of Xer recombination over ~40 generations were quite variable in different experiments, sometimes being lower and sometimes higher than the *dif*⁺ *RecA*⁺ control; nevertheless, the level of Xer recombination in the *dif*⁻ strain was always higher than that in the *RecA*⁻ *dif*⁺ strain (Figure 1). Therefore, chromosomal *dif* is not required for Xer recombination at plasmid *dif*.

The C-terminal domain of *FtsK* is required for Xer recombination at plasmid-borne *dif*, but not at the plasmid sites *cer* and *psi*

Steiner *et al.* (1999) have demonstrated that Xer recombination at *dif* in its normal chromosome position is dependent on the presence of *FtsK* protein with an intact C-terminal domain. In order to test whether the C-terminal domain of *FtsK* is required for Xer recombination at natural plasmid sites, at *dif* in different contexts, and at variant recombination sites, Xer recombination was analysed in strains with altered *FtsK* expression.

Plasmids containing directly repeated complete *cer* or *psi* sites were efficiently converted to resolved product in ~40 generations in an *FtsK*⁺ strain and in a strain carrying the *ftsK::cat1* allele, which expresses only the essential N-terminal domain I of *FtsK* as a consequence of the insertion of a chloramphenicol resistance cassette at nucleotide 678 of *ftsK* (Draper *et al.*, 1998; Figure 3A). We refer to this protein as *FtsK*_C⁻, since the available data indicate that the mutant phenotype arises from deletion of the C-terminal domain III. In contrast to the *FtsK*-independent Xer recombination at *cer* and *psi* sites, the plasmid pSDC124 containing directly repeated *dif* sites underwent very little Xer recombination in the *FtsK*_C⁻ strain (Figure 3A); this residual *FtsK*-independent recombination was abolished by inactivation of XerC. An unrelated low-copy-number plasmid derived from pSC101 containing directly repeated *dif* sites, pFC238, was also tested and showed no Xer recombination in the *FtsK*_C⁻ strain (data not shown), showing that the *FtsK*-dependency of

Fig. 3. (A) Xer recombination at plasmid-borne *dif*, *cer* and *psi* sites in $FtsK^+$ and $FtsK^-$ strains. Analysis of recombination products followed transformation of plasmids containing two directly repeated recombination sites into DS941 ($Xer^+ FtsK^+$), DS9041 ($FtsK^-$) and DS981 (Xer^-). Plasmid DNA was electrophoresed before and after digestion with *EcoRI*, which linearizes the plasmid backbone. The experiments shown here were carried out in a $RecF^-$ background; identical results were obtained in $RecF^+$ strains (data not shown). S_S , supercoiled substrate plasmid; S_L , linear substrate; RP_S , supercoiled resolved product; RP_L , linear resolved product. Bands running behind supercoiled substrates represent linear and dimeric forms of the plasmids, respectively. (B) Recombination at plasmid *dif* sites in the presence of overexpressed FtsK and FtsK-deletion derivatives. Boxes above gels indicate the portion of FtsK expressed from the complementing plasmid. Strains containing pBADK were maintained on media with glucose to suppress FtsK expression and subsequently transformed with the reporter plasmid, pFC238. The double transformants were then grown in the presence of either glucose or arabinose, as indicated. M indicates reporter plasmid, in the absence of pBADK. The effect of plasmid pAD12, expressing an N-terminally deleted FtsK derivative, on Xer recombination was tested using the *dif* reporter plasmid pFC238. Since pAD12 migrates at a position that obscures pFC238, the latter's presence was verified by restriction endonuclease digestion (data not shown). In the presence of pAD15, expressing domains I and II of FtsK, the reporter plasmid was pSDC124.

recombination at *dif* is independent of plasmid background and copy number.

The defect in Xer recombination at plasmid *dif* in the $FtsK^-$ mutant was efficiently complemented by wild-type FtsK protein expressed from the arabinose-controlled pBAD promoter of plasmid pBADK (Figure 3B; Guzman *et al.*, 1995; Liu *et al.*, 1998). Overexpression of FtsK from pBADK in the wild-type strain led to a higher level of Xer recombination than was observed in glucose-grown cells, or in pBADK-free cells. This suggests that the level of FtsK present in the wild-type strain is limiting for Xer recombination between *dif* sites in a multicopy plasmid. A pUC19-derived plasmid that is reported to express the C-terminal domain of FtsK cytoplasmically (pAD12, missing the 42 N-terminal amino acids; Diez *et al.*, 1997) was unable to complement the defect in Xer recombination in the $FtsK^-$ mutant (Figure 3B), suggesting that FtsK must be membrane- and septum-located for its function in Xer recombination. Although we have not demonstrated FtsK-related protein expression from pAD12, Diez *et al.* (1997) and Yu *et al.* (1998a) have reported that this plasmid produces a truncated FtsK with some residual function. A low-copy pSC101-derived plasmid expressing

all but the 65 C-terminal residues of FtsK from the normal chromosomal *ftsK* promoter (pAD15; Diez *et al.*, 1997) also failed to complement the defect in Xer recombination in the $FtsK^-$ strain (Figure 3B), confirming that it is the C-terminus of FtsK that is required for Xer recombination at *dif*. Furthermore, the presence of pAD15 resulted in severe inhibition of plasmid *dif* recombination in an $FtsK^+$ strain, demonstrating that this protein has a dominant-negative phenotype in Xer recombination. This suggests that FtsK function requires the association of two or more FtsK protomers, or that there are limited membrane sites at the septum for functional FtsK occupation. Consistent with the dominant-negative inhibition of *dif* recombination, cytological examination of $FtsK^+$ cells containing pAD15 showed a chromosome segregation defect similar to that observed in $FtsK^-$ cells (data not shown; see below).

To determine if the FtsK-independent recombination exhibited by *cer* and *psi* is a consequence of their requirement for host-encoded accessory proteins, or an intrinsic property of their core site sequence, we tested the FtsK-dependence of Xer recombination on a range of Xer recombination core sites that differ from *dif* in their nucleotide sequence (Figure 4A). Recombination at the

Fig. 4. Xer recombination at variant sites. (A) Sequence comparison of the core recombination sites *dif*, *dib*, *cer6* and *psi*. All sites possess a 6 bp central region (bold) flanked by 11 bp XerC- and XerD-binding sites. For *dib*, *cer6* and *psi* only nucleotides that distinguish these sites from *dif* are shown. (B) Recombination at variant sites. Plasmid DNA was isolated from *Xer*⁺ *FtsK*⁺ (DS941), *FtsK*⁻ (DS9041) and *XerC*⁻ (DS981) strains. Bands running behind the substrates (S) represent multimeric forms of the plasmids. In the case of the plasmid containing the *cer6* site without accessory sequences, Xer-mediated resolution generates two product circles (RP) of similar size, with the larger circle being competent to replicate. (C) Recombination in *dif*- and *dib*-containing plasmids. Strains were as described in (B). S₁ and S₂ represent the *dif*-containing (pSDC124) and *dib*-containing (pOW4) substrates, respectively. The resolution products (RP) generated by Xer recombination within both of these plasmids are identical in size. S_S and RP_S indicate supercoiled plasmids; S_L and RP_L denote *NdeI*-restricted plasmids. Note the incomplete digestion of the *dif*-containing plasmid from the *FtsK*⁺ strain.

psi core site was less efficient than that observed at the full *psi* site, but nevertheless remained *FtsK*-independent (compare Figure 4B with Figure 3A). Recombination at the *cer6* core site, which undergoes both intra- and intermolecular recombination (Summers, 1989), was also *FtsK*-independent, as was intramolecular resolution when it was juxtaposed to the *cer* accessory sequences (Figure 4B). Furthermore, addition of *psi* accessory sequences to a position adjacent to the XerC-binding site of *dif* resulted in a site that could undergo *FtsK*-independent resolution but was still dependent on *FtsK* for Xer-mediated multimerization (Figure 4B). These data indicate how sensitive *FtsK*-dependent recombination is to core site sequence, and demonstrate the ability of accessory sequences and proteins to bypass the *FtsK*-dependence of Xer intramolecular recombination at *dif*.

Mutations at *dif* and in the Xer recombination machinery can lead to *FtsK*-independence

In an attempt to ascertain if *FtsK*-dependence or -independence is determined at or prior to the initiation of recombination or at the processing of HJ recombination intermediates, the *FtsK*-dependence of recombination at an artificial site, *dib*, was analysed. This site is identical to *dif* with the exception of two transversions in the central region which make the strand cleaved by XerD purine rich (5/6 purines; Figure 4A). This site, when present in a synthetic HJ substrate, is predicted to adopt a conformation favourable for XerD-mediated strand exchange and

undergoes increased XerD strand exchange when compared with *dif* (Arciszewska *et al.*, 1997; Azaro and Landy, 1997; L.K.Arciszewska and D.J.Sherratt, unpublished data). Therefore, if *FtsK* acts by facilitating a change in HJ conformation, after initiation of catalysis by XerC, a *dib*-containing HJ intermediate may more readily adopt a conformation suitable for XerD strand exchange than a *dif*-containing HJ, and therefore recombine independently of *FtsK* (see Discussion).

In a wild-type background, plasmid pOW4, containing two directly repeated *dib* sites, undergoes less Xer recombination than the *dif*-containing plasmid pSDC124 (Figure 4C). In the *FtsK*⁻ strain, recombination between *dif* sites was barely detectable (<4% of total plasmid DNA present as recombinant product as compared with ~50% in the *FtsK*⁺ strain). In contrast, the level of recombination between *dib* sites was similar in the *FtsK*⁺ and *FtsK*⁻ backgrounds (~26% product and 20% product, respectively), consistent with *FtsK* functioning by facilitating a conformational change of the HJ intermediate, thereby allowing XerD-mediated resolution of HJs formed by XerC.

It has been proposed that the failure to observe recombination between *dif* sites *in vitro* reflects the requirement for an *in vivo* control that activates Xer recombination in cells containing chromosome dimers (Neilson *et al.*, 1999). *Haemophilus influenzae* XerC (HinXerC) together with *E.coli* XerD recombine supercoiled *dif* sites *in vitro* (Neilson *et al.*, 1999) suggesting that these recombinases

Fig. 5. FtsK-independent *dif* recombination in the presence of HinXerC. Strains AB1157 (Xer^+ $FtsK^+$), DS8029 ($XerC^-$) and GR51 ($XerC^-$ $FtsK_c^-$) were transformed with either pLMN132 (HinXerC⁺) or pSDC107 (EcoXerC⁺), followed by the *dif* reporter plasmid pSDC124. S, reporter plasmid substrate; RP, replicative resolved product. The arrow indicates the presence of an Xer-dependent multimeric species containing three *dif* sites.

can bypass the control mechanism. If this control requires FtsK function, we reasoned that *in vivo* recombination at *dif* may be rendered FtsK-independent if HinXerC replaces EcoXerC. We compared the ability of plasmid-expressed HinXerC and EcoXerC to support FtsK-independent *dif* recombination in pSDC124 (Figure 5). Both of these recombinases were able to complement efficiently the *dif* recombination defect in the $XerC^-$ strain. However, in the $XerC^-$ $FtsK_c^-$ strain a low level of recombination (~8% resolved product in addition to a multimeric plasmid dependent on Xer recombination) was observed in the presence of HinXerC, whereas no detectable Xer recombination was observed in the presence of EcoXerC. We conclude that the ability of HinXerC to recombine supercoiled *dif*-containing substrates *in vitro* reflects an ability to bypass the FtsK-dependency of Xer recombination at *dif*, perhaps by promoting a HJ conformation suitable for XerD-mediated resolution.

Xer recombination between plasmid-borne *dif* and chromosomal *dif* is dependent on the C-terminal domain of FtsK

The FtsK-dependence of intermolecular Xer recombination between chromosomal *dif* and plasmid *dif* was analysed using a pSC101 replicon temperature-sensitive for DNA replication. Recombination was assayed with *dif* located in its normal position at the replication terminus (at ~1589 kb) and at an ectopic position, within *lacZ* (at 365 kb), in both $FtsK^+$ and $FtsK_c^-$ strains (Table I). Strains deleted for chromosomal *dif* support negligible plasmid integration, confirming that the integration frequency is a reflection of *dif* recombination activity. In strains containing *dif* in its normal location, the level of plasmid integration was >350-fold lower in the $FtsK_c^-$ strain than in the $FtsK^+$ parent. When *dif* was translocated to *lacZ*, the integration frequency was still FtsK-dependent although the difference in frequency between the $FtsK^+$ and $FtsK_c^-$ strains was reduced. The frequency of integra-

tion in the absence of the FtsK C-terminus is similar whether *dif* is located at its normal site or at an ectopic site, suggesting that this represents a basal level of FtsK-independent and position-independent Xer recombination.

The cytologically visible defects of chromosome segregation in an $FtsK_c^-$ strain are attributable to the failure to resolve chromosome dimers by Xer recombination

In order to determine how much of the cytologically visible defect in chromosome segregation in $FtsK_c^-$ strains arises from a failure of Xer recombination at *dif* to convert dimers to monomers, we analysed a *RecA*⁻ derivative of an $FtsK_c^-$ strain. Cells were grown in minimal medium in order to maintain the generation time at >60 min so that cells should contain 1–2 chromosomes. The $FtsK_c^-$ strain filamented extensively, forming septate and aseptate filaments with aberrantly segregated and located nucleoids (Figure 6A). More than 50% of the potential colony-forming units were septate or aseptate filaments, with septate chains predominating. Cells of a *RecA*⁻ derivative of this strain (Figure 6B) more closely resembled the $FtsK^+$ *RecA*⁻ strain (Figure 6C) than the $FtsK_c^-$ *RecA*⁺ strain with respect to the proportion of filamentous cells (<10% of potential colony-forming units) and the nature of the filaments. There was a complete absence of cell chains with nucleoids trapped by invaginating septa and the few aseptate filaments present were either anucleate or contained well segregated nucleoids. This indicates that the phenotype arising from deletion of the FtsK C-terminus is primarily a result of a failure to resolve chromosome dimers by Xer recombination; impairment of dimer formation suppresses this defect. Consistent with this we have observed that *lexA3 dif*⁻ cells, which cannot induce the SOS response, resemble $FtsK_c^-$ strains, forming chains in which septa appear to dissect entrapped chromosomes (Figure 6E). Furthermore, *lexA3 FtsK_c^-* cells had a phenotype similar to *LexA*⁺ $FtsK_c^-$ cells, demonstrating that the loss of chromosome segregation phenotype in the $FtsK_c^-$ *RecA*⁻ strain is not a consequence of a failure to induce the SOS response (data not shown).

Chromosome positioning in cells depleted for FtsZ and in septate chains unable to resolve chromosome dimers

Placement of FtsK at the septum requires FtsZ (Wang and Lutkenhaus, 1998; Yu *et al.*, 1998b). Therefore it follows that if cells are unable to undergo cell division due to a deficiency of FtsZ, they may well have a chromosome segregation defect, as dimers generated by homologous recombination cannot be resolved by Xer recombination, because of the failure to localize FtsK to the septum. Our microscopic observations support this (Figure 6F and G). Combined fluorescence–phase contrast images of mid-log phase $FtsZ^{ts}$ *RecA*⁺ cells that had been shifted to 42°C for 90 min show that in *FtsZ*-depleted filaments, nucleoids are aberrantly positioned and many appear multimeric (Figure 6F). We propose that this results from a failure to resolve chromosome dimers by FtsK-dependent Xer recombination, and hence a failure to segregate chromosomes properly. In $FtsZ^{ts}$ *RecA*⁻ cells, in which chromosome dimers should not form, no chromosome segregation defect is apparent and nucleoids appear well separated

Table I. Frequencies of integration of a *dif*-containing plasmid into the *E.coli* chromosome

Strain	Relevant genotype/phenotype	Integration frequency	
		Mean	Standard deviation
AB1157	Xer ⁺ FtsK ⁺	6.8×10^{-2} (100)	2.1×10^{-2}
DS941	AB1157 <i>recF</i>	4.9×10^{-2} (72)	2.7×10^{-2}
NL40	AB1157 <i>recF difΔ6</i>	2.9×10^{-6} (0.004)	3.4×10^{-6}
NL250	AB1157 <i>recF difΔ6 lacZ::dif</i>	6.6×10^{-4} (0.97)	6.0×10^{-4}
DS9041	AB1157 <i>recF ftsK</i>	1.3×10^{-4} (0.19)	1.1×10^{-4}
MA1	AB1157 <i>recF difΔ6 ftsK</i>	5.2×10^{-7} (0.0008)	7.1×10^{-7}
MA2	AB1157 <i>recF difΔ6 lacZ::dif ftsK</i>	1.1×10^{-4} (0.16)	1.0×10^{-4}

The *dif*-containing plasmid used was pFH127. Frequencies shown are the averages of three independent determinations and are calculated as the ratio of Sp^R bacterial colonies at 42°C to those at 30°C. The relative frequencies of integration in mutant strains compared with AB1157 (100%) are given in parentheses.

Fig. 6. Cytological examination of chromosome positioning and segregation. In the combined fluorescence–phase contrast images shown, nucleoids appear green. Scale bars are 2 μm in (A–E), and 5 μm in (F and G).

(Figure 6G). The clear separation of the nucleoids in this *RecA*[−] strain allows the estimation of chromosome number. Analysis of 569 *FtsZ*^{ts} *RecA*[−] filaments with eight or less chromosomes revealed that ~55% contained less than the 2ⁿ chromosomes that would result from synchronous initiation of chromosome replication in the absence of chromosome loss; the overall frequency of chromosome loss was ~25%. This is comparable to the frequency of chromosome loss in a *RecA*[−] strain determined by flow cytometry (Skarstad and Boye, 1993). The even positioning and spacing of chromosomes in the *FtsZ*^{ts} *RecA*[−]

strain (Figure 6G) indicate that an intact septal ring need not play a role in correct nucleoid positioning.

In the *FtsK*_c[−] strain we have noticed that septate chains of four and eight cells frequently have a centrally placed, entrapped, apparently dimeric nucleoid with symmetrically and evenly spaced monomeric nucleoids on either side (Figure 6A, arrows). The monomeric nucleoids are mispositioned with respect to the septa, the DAPI-stained material being immediately adjacent to the septum. It appears that an initial dimeric chromosome bisected by a septum directs the even positioning of the other nucleoids

and hence their misplacement with respect to the septa. This observation underlines the ability of nucleoid and septal positioning to be independent of each other; in the septate chains, nucleoid and septum mis-positioning appear to be determined independently by the initial attempt to place a septum that bisects a dimeric chromosome. We believe it likely that septation adjacent to the apparent monomeric chromosomes is incomplete because some region of each chromosome is entrapped on the other side of the septum. The origins of these four and eight chromosome-equivalent septate chains with a centrally placed apparent dimer remain a mystery for cells whose mean generation time is ≥ 60 min, although replication of a HJ-containing partial dimer could generate the observed pattern.

Discussion

A combination of genetic, biochemical and cytological techniques is providing new insight into the processes of chromosome segregation, cytokinesis and cell division in bacteria (reviewed in Levin and Grossman, 1998; Sharpe and Errington, 1999). Furthermore, the importance of interplay between DNA replication and recombination is emerging (Cox, 1998). The work presented here extends earlier experiments of Steiner *et al.* (1999) and highlights the role of FtsK in linking cell division with chromosome segregation, replication and recombination.

We propose that the C-terminal domain of FtsK may have two roles in chromosome segregation. First, FtsK may 'pump' newly replicated chromosomes away from the septal space during septation, by an uncharacterized mechanism; this could be equivalent to the process that the related protein, SpoIIIE, performs during *B.subtilis* sporulation (Wu *et al.*, 1995). If this process fails to remove DNA from the septal region, e.g. when chromosomal dimers are present, then FtsK mediates its second role, in Xer recombination at *dif*. It is impairment of this second role that leads to most of the observed cytological defects in chromosome segregation, since preventing the formation of chromosomal dimers by introducing a *recA* mutation suppresses the visible chromosome segregation defect of the FtsK_c⁻ strain.

Our favoured explanation for the FtsK- and RecA-dependence of Xer recombination at *dif* is that this recombination only occurs when chromosomal dimers form by homologous recombination. During the attempted segregation of these dimers, the action of the C-terminal domain of FtsK leads to Xer-mediated conversion of dimers to monomers, thereby allowing chromosome separation and segregation. Implicit in this process is an activation step that arises as a consequence of the failure to segregate a chromosomal dimer. Such a mechanism would help ensure that Xer recombination at chromosomal *dif* is restricted to converting dimers to monomers, with little or no *dif* recombination occurring in cells that contain monomers (Figure 7A). It would also ensure that Xer recombination occurs at the appropriate stage in the cell cycle, immediately prior to cell division. Consistent with this need for activation is the observation that Xer recombination at *dif* *in vitro* on duplex DNA has not been observed with the *E.coli* recombinases XerC and XerD, whereas these same recombinases efficiently recombine

Fig. 7. (A) Xer recombination at *dif* during the *E. coli* cell cycle. The fate of a replicating chromosome is depicted in the presence and absence of a single homologous recombination event. Filled circles represent origins of replication and triangles represent *dif* sites. (B) Model of Xer site-specific recombination reactions on *cer*, *psi* and *dif*. XerC initiates recombination on supercoiled substrates to form an HJ intermediate. With *cer*-containing molecules, the HJ nucleoprotein complex dissociates, since HJs accumulate *in vivo* and *in vitro* (McCulloch *et al.*, 1994; Colloms *et al.*, 1996). These HJ intermediates are processed by an Xer-independent pathway *in vivo*. With *psi*-containing molecules, the HJs are recombined by XerD (Colloms *et al.*, 1996), presumably after the indicated conformational change (see Gopaul *et al.*, 1998). For *dif* we propose that in the absence of the C-terminal domain of FtsK, any XerC-promoted HJ intermediates are efficiently reversed to substrate by XerC. The proposed action of FtsK is to facilitate the conformational change that will generate a substrate suitable for XerD strand exchange. This could occur directly through FtsK action on the HJ or indirectly through FtsK-mediated alteration of chromosomal or plasmid architecture. We propose that Xer recombination at *dif* becomes FtsK-independent when *psi* accessory sequences are placed adjacent to *dif* because the XerC-generated HJs behave like those of *cer*. This is supported by the demonstration that HJ intermediates, but not complete recombinant products, form efficiently by Xer recombination on supercoiled substrates containing *dif* sites with adjacent *psi* accessory sequences (B.Hallet, O.Waissbein and D.J.Sherratt, unpublished data).

the plasmid sites *cer* and *psi* in the presence of the appropriate accessory proteins (Colloms *et al.*, 1996, 1997).

We believe it likely that chromosomal dimers are the normal substrate for Xer recombination at *dif*. HJ-containing intermediates of homologous recombination may also become Xer recombination substrates if the HJs are translocated to *dif*; this might occur most often when homologous recombination is initiated close to *dif*, as occurs frequently (Corre *et al.*, 1997). If homologous

recombination-generated HJ intermediates were the normal substrate for Xer recombination, it would explain why Xer recombination at chromosomal *dif* only occurs under conditions that permit homologous recombination. However, HJs formed by homologous recombination are unlikely to be the substrates for the Xer recombination events assayed here, since this would involve the HJ branch migrating over at least 1.3 kb of heterology. Furthermore, the frequency of Xer recombination at plasmid *dif* is unperturbed in RecF⁻ strains, which are deficient in plasmid homologous recombination yet competent in chromosomal homologous recombination (Kolodner *et al.*, 1985). It therefore appears that the apparent requirement for chromosomal dimers for activation of Xer recombination at plasmid *dif* is by a mechanism other than through the provision of a HJ substrate.

It seems likely that in RecA⁺ cells chromosomal dimers are present at the time of initiation of cell division at some substantial frequency, largely as a consequence of recombinational repair of stalled or broken replication forks (e.g. see Cox, 1998). We observed that ~25% of the nucleoids have been lost in FtsZ^{ts} RecA⁻ filaments, presumably as a consequence of premature stalling or breakage of replication forks. Our results on nucleoid positioning in FtsZ^{ts} filaments and the failure of a plasmid encoding domains II and III of FtsK to complement the Xer recombination defect of an FtsK_c⁻ strain, suggest that chromosomal or plasmid *dif* sites and their associated recombination machinery are positioned in the vicinity of FtsK and the invaginating septum during FtsK-dependent Xer recombination. Activation of Xer recombination may require only the opportunity for *dif* site synapsis in the region of septum-associated FtsK. The synapsis of chromosomal *dif* sites may occur efficiently only when *dif* is positioned in the terminus region, because as yet uncharacterized aspects of cellular chromosome biology impose restraints on which homologous chromosomal sequences can physically interact in the region of the septum. For example, the late replication of *dif* and the probable location of the DNA replication machinery in the region of the septum (Lemon and Grossman, 1998) might facilitate the co-localization of two chromosomal *dif* sites, their associated recombinases and FtsK. Similarly, replication of *dif*⁺ plasmids at a septum-proximal replication machine might facilitate their access to FtsK. The reduced Xer recombination activity of an ectopic *dif* site inserted into *lacZ* may reflect the site's reduced access to the septum, as implied by the FtsK-dependency of most of the residual Xer recombination. The observation that Xer recombination between plasmid-borne *dif* sites also requires conditions that generate chromosomal dimers in addition to the C-terminal domain of FtsK, indicates that activation of Xer recombination at *dif* requires additional processes that occur before, at, or after the FtsK-dependent step. These could include modification of the activity or amount of FtsK or the recombinases. Alternatively, the requirement for chromosomal dimers for Xer recombination at plasmid *dif* sites might arise because the presence of dimers leads to a cell division time-lag that increases the opportunity for plasmids to become associated with the septum and FtsK. Whatever this activation process is, it appears to be necessary for Xer recombination at *dif* sites present in both the chromosome and on plasmids.

Furthermore, it provides a mechanism for resolution selectivity which is fundamentally different to the topological filter that acts during Xer recombination at *cer* and *psi* (Colloms *et al.*, 1996, 1997), and during resolvase/invertase-mediated recombination (Stark and Boocock, 1995). In the latter cases there are topological restraints on the formation of productive synaptic complexes arising from intermolecular interactions, whereas in Xer recombination at *dif* it appears that the appropriate substrates and enzyme machinery only become accessible to each other when chromosomal dimers form; there is no intrinsic mechanistic barrier to intermolecular recombination at *dif*, as demonstrated by the integration of *dif*-containing plasmids into the chromosome and the multimerization of plasmids containing *dif*.

The observations that the plasmid recombination sites *cer* and *psi* recombine independently of FtsK and RecA, while addition of *psi* accessory sequences to *dif* makes its intramolecular recombination independent of FtsK, highlight the subtle nature of the FtsK- and RecA-dependence and how this dependency can be bypassed by accessory sequences and proteins. Furthermore, the observation that an XerC homologue from *H.influenzae*, as well as mutation in the *dif* core site, promotes FtsK-independence reinforces these conclusions and suggests a mechanism by which FtsK may act (Figure 7B). We propose that FtsK functions by facilitating a conformational change in the HJ intermediate formed by XerC, converting it from a form that favours XerC strand exchange to one favouring XerD strand exchange. Previous work has indicated that HJ conformation and resolution can be altered by changes in external topology (Zerbib *et al.*, 1997). In this way, FtsK and the invaginating septum may provide the 'catalyst' for the completion of the Xer recombination reaction and hence the conversion of dimers to monomers. Following dimer resolution, the physical separation of the monomer chromosomes provides a barrier to intermolecular Xer recombination. Although we favour this model of FtsK action, we cannot eliminate the possibility that FtsK acts earlier in the recombination pathway.

The results presented here consolidate the view that there is an effective coordination between the different aspects of DNA metabolism and the cell cycle. FtsK may play a key role in linking cell division (through its N-terminal domain) and the segregation of chromosomes (via its C-terminal domain). Yu *et al.* (1998a) have proposed some shared functionality between FtsK and MukB, another protein implicated in chromosome segregation. MukB⁻ strains are not impaired in Xer recombination at *dif* (our unpublished data). The high correspondence between the presence of Xer-like sequences and FtsK-related sequences in any given bacterium (G.D.Recchia, unpublished observations) may reflect a functional conservation of the Xer-FtsK interaction.

Materials and methods

Bacterial strains and plasmids

Escherichia coli strains and plasmids are listed in Table II.

Growth conditions

Cells were grown in LB medium containing 1% NaCl, with the exception of those containing the *ftsZ84* allele which were grown in LB containing

Table II. Strains and plasmids

Strain	Relevant genotype/description ^a	Source/reference
AB1157	Rec ⁺ laboratory strain	Summers and Sherratt (1988)
DS931	<i>AB1157 lexA3</i>	laboratory stock
DS941	<i>AB1157 recF lacI^q</i>	Summers and Sherratt (1988)
DS8029	<i>AB1157 xerC</i>	this study
MA3	<i>AB1157 ftsK</i>	this study
GR51	<i>AB1157 xerC ftsK</i>	this study
GR35	<i>AB1157 difΔ6</i>	this study
GR19	<i>AB1157 recA938</i>	this study
GR58	<i>AB1157 recA56 srl::Tn10</i>	this study
GR59	<i>AB1157 ftsK recA56 srl::Tn10</i>	this study
GR47	<i>DS931 difΔ6</i>	this study
DS981	<i>DS941 xerC</i>	Colloms <i>et al.</i> (1990)
RM40	<i>DS941 lacPO-xerC</i>	McCulloch <i>et al.</i> (1994)
DW1	<i>DS941 lacPO-xerC recA938</i>	this study
NL40	<i>DS941 difΔ6</i>	Leslie and Sherratt (1995)
NL250	<i>DS941 difΔ6 lacZ::dif</i>	Leslie and Sherratt (1995)
DS9041	<i>DS941 ftsK</i>	this study
MA1	<i>DS941 difΔ6 ftsK</i>	this study
MA2	<i>DS941 difΔ6 lacZ::dif ftsK</i>	this study
MCZ84	<i>MC4100 leu::Tn10 ftsZ84</i>	Bi and Lutkenhaus (1992)
GR14	<i>MCZ84 recA938</i>	this study
Plasmid	Description ^b	Source/reference
pSDC124	pUC18-based <i>dif</i> reporter	Blakely <i>et al.</i> (1991)
pFC238	pGB2-based <i>dif</i> reporter	F.Cornet
pOW4	pUC19-based <i>dib</i> reporter	D.J.Sherratt
pSDC115	pBR322-based <i>cer</i> reporter	McCulloch <i>et al.</i> (1994)
pSDC134	pUC18-based <i>psi</i> reporter	Colloms <i>et al.</i> (1996)
pNAG301	pUC18-based <i>psi</i> (core) reporter	N.Ganguly
pSDC110	pBR322-based <i>cer6</i> (core) reporter	S.Colloms
pSDC162	pUC18-based <i>cer6</i> reporter	S.Colloms
pBH202	pUC18-based <i>dif</i> core:: <i>psi</i> accessory sequences reporter	B.Hallet
pFH127	pGB2ts containing a single <i>dif</i> site	Hayes and Sherratt (1997)
pBADK	pBAD18 expressing full-length FtsK from pBAD promoter	Draper <i>et al.</i> (1998)
pAD12	pUC19 expressing N-terminal truncated FtsK	Diez <i>et al.</i> (1997)
pAD15	pGB2 expressing C-terminal truncated FtsK from normal chromosomal promoter	Diez <i>et al.</i> (1997)
pSDC107	pCB104 expressing <i>E.coli</i> XerC	S.Colloms
pLMN132	pACYC184 expressing <i>H.influenzae</i> XerC	L.Neilson

^aStrains generated in this study were constructed by P1 transduction. *recA* mutants were confirmed as RecA⁻ by UV-sensitivity assays. Strains carrying the temperature-sensitive allele *ftsZ84* were verified by their inability to form colonies on solid media at 42°C and by filamentation in liquid media at 42°C.

^bReporter plasmids contain two directly repeated recombination sites.

0.4% NaCl, to enhance the mutant phenotype. For depletion of FtsZ, *ftsZ84* cells were grown to mid-log phase at 30°C and then shifted to 42°C. Minimal M9 medium containing 0.2% glucose and casamino acids (0.5%) or essential amino acids was generally used for cytological examination of FtsK⁻ strains. Mean generation time was ~80 min under these conditions, which consistently produced less complex and more reproducible phenotypes than LB (mean generation time ~25 min), probably as chromosome number is reduced under slower growth conditions. Glucose (0.2%) was used for repression of appropriate plasmid and chromosomal promoters. L-arabinose (0.2%) was added to media for induction of the promoter in pBADK, and 2 mM IPTG was used for the induction of chromosomal *lac* promoters.

In vivo plasmid recombination assays

For assays of Xer recombination *in vivo*, plasmids containing two directly repeated recombination sites were transformed into the relevant strains and 20–30 pooled transformant colonies were grown overnight. Plasmid DNA was isolated using QIAprep kits (Qiagen) and treated with appropriate restriction enzymes. DNA was analysed by electrophoresis through 1% agarose TAE gels and visualized by staining with SYBR green I (Flowgen). Gels were scanned using a Molecular Dynamics Fluorimager 575. ImageQuant software (Molecular Dynamics) was used for quantitation of bands.

In vivo time course recombination assays using RM40 and its derivative were performed as described by McCulloch *et al.* (1994). To ensure that XerC expression was sufficiently repressed, cells were grown in media containing 0.2% glucose at all stages prior to induction, both before

and after transformation with the relevant reporter plasmid. Pooled transformant colonies (20–40) were grown to mid-log phase, when glucose was removed by centrifugation, after which cells were re-suspended in the same volume of pre-warmed LB containing 2 mM IPTG and grown for a further 3 h. Aliquots were taken at 30 minute intervals and plasmid DNA prepared from either 5 ml of culture (0 min) or 1.5 ml of culture (later time points).

Plasmid integration assays

The level of intermolecular recombination between a plasmid-borne *dif* site and chromosomal *dif* was assayed as described previously (Leslie and Sherratt, 1995; Hayes and Sherratt, 1997).

Fluorescence microscopy and image analysis

For microscopic analysis, cells from a stationary phase culture were diluted into fresh medium and grown to mid-log phase. Nucleoids were condensed with chloramphenicol (200 µg/ml) for 15 min prior to staining with DAPI (1 µg/ml). Phase-contrast and fluorescence images were acquired using a cooled CCD camera (Princeton Instruments) and Metamorph 3.5 image acquisition software (Universal Imaging Corporation) from an Olympus BX60 fluorescence microscope. Final images were transferred to Adobe Photoshop 5.0 for printing.

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