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Asymmetric activation of Xer site-specific recombination by FtsK

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Chromosome dimers, which frequently form in *Escherichia coli*, are resolved by the combined action of two tyrosine recombinases, XerC and XerD, acting at a specific site on the chromosome, *dif*, together with the cell division protein FtsK. The C-terminal domain of FtsK (FtsK$_C$) is a DNA translocase implicated in helping synapsis of the *dif* sites and in locally promoting XerD strand exchanges after synapse formation. Here we show that FtsK$_C$ ATPase activity is directly involved in the local activation of Xer recombination at *dif*, by using an intermolecular recombination assay that prevents significant DNA translocation, and we confirm that FtsK acts before Holliday junction formation. We show that activation only occurs with a DNA segment adjacent to the XerD-binding site of *dif*. Only one such DNA extension is required. Taken together, our data suggest that FtsK needs to contact the XerD recombinase to switch its activity on using ATP hydrolysis.

INTRODUCTION

Repair of circular DNA molecules by homologous recombination at stalled or broken replication forks can lead to crossing over and hence dimer formation (Cox *et al*, 2000; Michel *et al*, 2001). Bacteria containing chromosome dimers cannot segregate their DNA, or divide correctly, and form filaments before dying. Chromosome dimers form every seven generations on average in laboratory-grown *Escherichia coli*, so mechanisms that ensure that the chromosome is monomeric are central to bacterial survival

and growth (Steiner & Kuempel, 1998; Pérals *et al*, 2000). In *E. coli*, Xer site-specific recombination converts chromosome dimers into monomers by the addition of a crossover at a 28 base-pair (bp) recombination site, *dif*, located in the replication termination region of the chromosome. Recombination is mediated by two related tyrosine recombinases, XerC and XerD, within a nucleoprotein complex, (XerCD–*dif*)$_2$, which contains two molecules each of XerC and XerD and two synapsed *dif* sites. XerC and XerD catalyse two sequential pairs of DNA strand exchanges such that recombination proceeds via a Holliday junction (HJ) intermediate (reviewed in Barre & Sherratt, 2002).

Chromosome dimer resolution also requires FtsK, a large, multifunctional, integral membrane protein, which coordinates chromosome segregation and cell division (Liu *et al*, 1998; Capiaux *et al*, 2001). Such coordination is crucial in bacteria where DNA replication and segregation are not separated in time, and can occur as cells divide.

FtsK can be divided into three domains: a membrane-spanning N-terminal domain (FtsK$_N$), which localizes to the division septum and is essential for cell division (Draper *et al*, 1998); a long linker (600 amino acids) of unknown function; and a C-terminal AAA$^+$ ATPase domain (FtsK$_C$; Yu *et al*, 1998; Barre *et al*, 2000; Aussel *et al*, 2002). Two roles have been assigned to FtsK in chromosome dimer resolution. First, FtsK has been implicated in positioning the terminus regions of chromosome dimers at mid-cell and synapsing their *dif* sites (Capiaux *et al*, 2002; Corre & Louarn, 2002). Consistent with such a role, FtsK$_{50C}$, an active derivative of FtsK$_C$, was shown to be an ATP-dependent DNA translocase *in vitro* (Aussel *et al*, 2002). Second, FtsK$_C$ is directly involved in Xer recombination. *In vitro* analyses of FtsK-dependent Xer recombination between plasmid-borne *dif* sites suggest that FtsK$_{50C}$ can switch the catalytic state of (XerCD–*dif*)$_2$ so that recombination is initiated by XerD rather than XerC (Aussel *et al*, 2002). 'Mix and match' experiments between *Haemophilus influenzae* and *E. coli* Xer–FtsK, and the exploitation of FtsK chimaeras containing different segments of the C-terminal domains of *H. influenzae* and *E. coli* FtsK have shown that the C-terminal 140 amino acids of FtsK$_C$ interact with (XerCD–*dif*)$_2$ during recombination activation (Yates *et al*, 2003).

Is ATP hydrolysis by FtsK$_C$ necessary for local recombination activation, or is it only required for synapse formation between *dif* sites carried on long DNA molecules? We have analysed the local

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role of FtsK$_C$ in the activation of Xer recombination by using a new *in vitro* intermolecular Xer recombination assay that does not provide an opportunity for significant DNA translocation. We show that activation by FtsK$_{50C}$ still depends on ATP hydrolysis. Furthermore, the presence of nicks as close as 3 bp from the recombination site did not prevent recombination activation, supporting the idea that the motor domain of FtsK functions in the local activation of the recombinases independently of global DNA translocation. In addition, we show that Xer activation by FtsK$_{50C}$ requires only one duplex DNA arm protruding from the (XerCD–*dif*)$_2$ complex, adjacent to an XerD-binding site. This observation is the first evidence for asymmetry in the recognition of (XerCD–*dif*)$_2$ by FtsK, and suggests a specific interaction between the recombinase that carries out the first pair of strand exchanges, XerD, and the FtsK activator. This asymmetry implies that the orientation of *dif* sites with respect to the septum-localized FtsK must be nonrandom *in vivo*.

RESULTS

FtsK$_{50C}$ activates intermolecular Xer recombination

To analyse the local role of FtsK$_C$ in the activation of Xer recombination in the absence of significant DNA translocation, we developed an *in vitro* intermolecular recombination reaction between two short *dif*-containing DNAs. The lengths of the DNAs were chosen so as to preclude significant translocation by FtsK$_{50C}$ (Fig 1A). In initial experiments, one DNA molecule contained a *dif* site flanked by arms of 176 and 207 bp, whereas the second DNA molecule consisted of *dif* flanked by arms of 16 bp, and was 5′-end-labelled on both strands with ^{32}P. Incubation of equimolar concentrations of the two DNAs with XerCD in the presence of FtsK$_{50C}$ and ATP led to a complete intermolecular site-specific recombination reaction (Fig 1B). The reaction was absolutely

dependent on ATP hydrolysis by FtsK$_{50C}$, as nonhydrolysable AMP-PNP and Walker A and B box mutants of FtsK$_{50C}$, which cannot hydrolyse ATP, did not support recombination (Fig 1B and data not shown). The poorly hydrolysable nucleotide ATP-γS supported a low but detectable level of recombination, correlating with its expected rate of hydrolysis.

XerCD activation needs DNA on one side of *dif* only

Short unlabelled DNAs with either one or two *dif* flanking arms were reacted with a minimal ^{32}P-labelled *dif* site flanked on either side by just 3 bp of DNA (3CD3) to explore the requirements for FtsK$_{50C}$-dependent activation of XerCD recombination. Remarkably, an absolute asymmetry was observed: only unlabelled DNA fragments with a flanking arm adjoining the XerD-binding site of *dif* (D-side DNA) could be recombined with the minimal labelled fragment. No reaction was observed when the flanking DNA was adjoining the XerC-binding site (C-side DNA; Fig 2). This asymmetric requirement for flanking DNA indicates either that FtsK$_{50C}$ needs to activate the (XerCD–*dif*)$_2$ nucleoprotein complex from a position on DNA adjacent to bound XerD or that FtsK$_{50C}$ has a DNA sequence or structure dependence, with only a specific D-side DNA sequence permitting efficacious FtsK$_{50C}$ loading.

To distinguish these two possibilities, unlabelled DNAs with identical DNA sequences adjoining either the XerC- or XerD-binding sites of *dif* were compared for their ability to support FtsK$_{50C}$-dependent stimulation of XerCD–*dif* recombination.
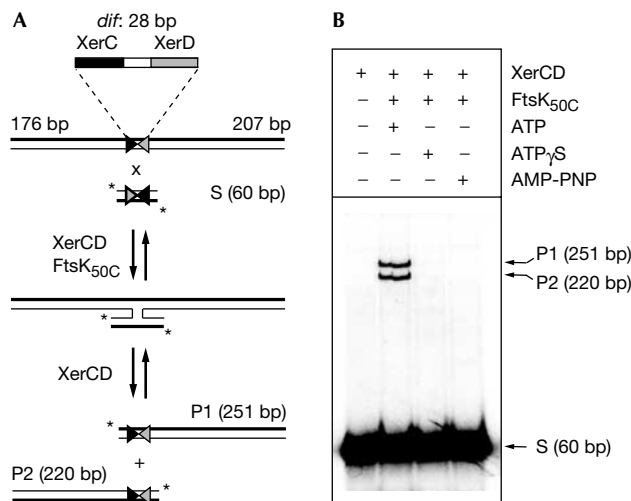
Fig 1 | Minimal XerCD–FtsK recombination system. (**A**) Schematic illustrating an intermolecular Xer recombination reaction between a 411 bp unlabelled DNA fragment containing a centrally located *dif* and a short, *dif*-containing DNA (60 bp) 5′-end-labelled with ^{32}P on both strands. (**B**) Intermolecular recombination by XerCD is absolutely dependent on FtsK$_{50C}$ and ATP. Reactions were incubated at 37 °C for 60 min and analysed by 7% TBE–PAGE.
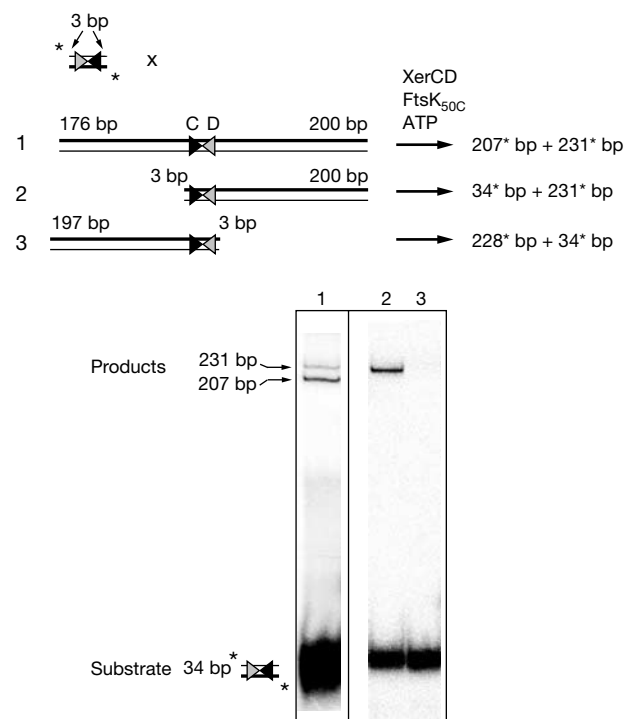
Fig 2 | FtsK-dependent Xer recombination requires DNA adjacent to the XerD-binding site only. The schematic illustrates reactions between short, labelled *dif*-containing DNAs and unlabelled DNAs with either (1) two arms flanking *dif* or one arm adjacent to *dif* on either the XerD side (2) or the XerC side (3). Recombination reactions were incubated at 37 °C for 60 min and analysed by 7% TBE–PAGE.

These DNA fragments were derived from two *dif*-containing plasmids that were identical apart from an inverted *dif* orientation (pFX315 and pFX316; Fig 3A). Only those DNA molecules with an arm adjoining the XerD-binding site of *dif* recombined with 3CD3 in an FtsK$_{50C}$-dependent reaction (Fig 3B). In total, three different D-side DNA sequence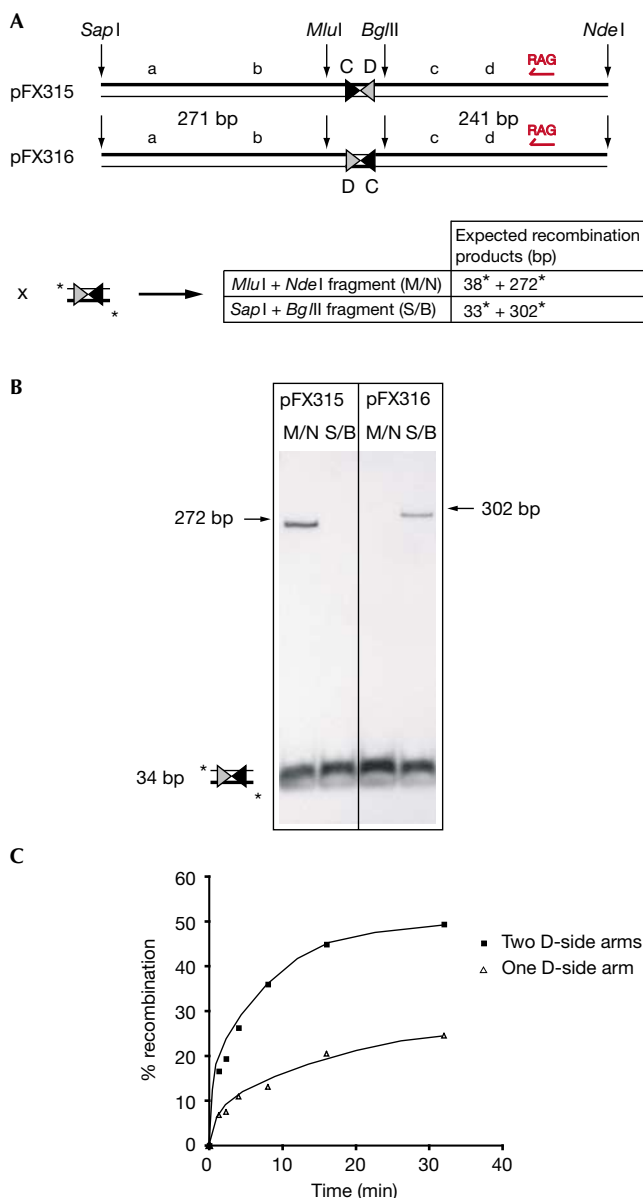s could support recombination. These data demonstrate that the presence rather than the sequence of D-side DNA is required for FtsK$_{50C}$ activation of Xer recombination.

A second asymmetry arises from the observation that only one D-side DNA segment is required for activation of Xer recombination in the intermolecular assay (Figs 2,3). Adding a second D-side arm into the reaction (on the labelled 3CD3 fragment) doubles the proportion of recombination without affecting the reaction profile, as expected if FtsK$_{50C}$ has only to approach the (XerCD–*dif*)$_2$ synaptic complex on one D-side arm to activate recombination (Fig 3C).

D-side DNA length affects recombination efficiency

Having shown that D-side DNA is essential for activation of Xer recombination by FtsK$_{50C}$, we investigated whether the length of this DNA arm affected the efficiency of the reaction. Unlabelled short fragments with various lengths of C- or D-side DNA extension were reacted with ^{32}P-labelled 3CD3 DNA or DNA with 3 bp on the C side and 16 bp on the D side ('3CD16'; Fig 4A). Increasing D-side DNA length from 48 to 207 bp increased the level of labelled recombinant product to a maximum of approximately 10% (Fig 4A,B). Further increases in DNA length did not increase the levels of recombination observed (data not shown). As expected, none of the DNAs with extensions only on the C side of *dif* reacted. However, adding a short 16-bp arm on the D side of the labelled fragment enabled recombination to occur at a low level independently of unlabelled C-side arm length, presumably due to inefficient FtsK$_{50C}$ loading on the 16-bp D-side arm (Fig 4A, right panel).

Why does FtsK$_{50C}$ activation of Xer recombination exhibit such a DNA length dependence? Examination of FtsK$_{50C}$ ATPase activity as a function of DNA length (Fig 4C) demonstrates a biphasic relationship between ATPase level and DNA length. The first phase shows a linear relationship between ATPase activity and DNA length, as DNA length increases from 0 to ∼80 bp. The second phase, of shallower gradient, shows that further increases in DNA length lead to only small increases in ATPase turnover. The DNA length threshold of 80 bp could reflect either the amount of DNA required to support stable and productive FtsK$_{50C}$–DNA interaction, or the length of DNA translocated in a single catalytic cycle (if ATP hydrolysis is correlated with translocation). We cannot distinguish between these two possibilities, although the observation that a circular plasmid stimulates ATP hydrolysis by FtsK$_{50C}$ up to four times as strongly as when it is linear (data not shown) suggests that FtsK$_{50C}$ translocates processively in steps of more than 80 bp. Also, related AAA$^+$ ring proteins, such as RuvB and MCM, cover between 20 and 70 bp DNA per active oligomer (Chen *et al*, 2002; Fletcher *et al*, 2003), so the 80-bp ATPase threshold for FtsK$_{50C}$ could reflect the FtsK$_{50C}$ 'footprint' of a single FtsK$_{50C}$ oligomer. The DNA length threshold of 150–200 bp for recombination could indicate that more than a single FtsK$_{50C}$ oligomer is required for recombination activation.

D-side DNA nicks do not prevent Xer activation

As FtsK$_{50C}$ can generate superhelical torsion in DNA as it translocates, we reasoned that even the local activation of Xer recombination by FtsK$_{50C}$ might require the continuity of the phosphodiester backbone on the D-side DNA. Addition of a single-strand nick at various positions on the DNA segment adjacent to the XerD-binding site did not compromise the
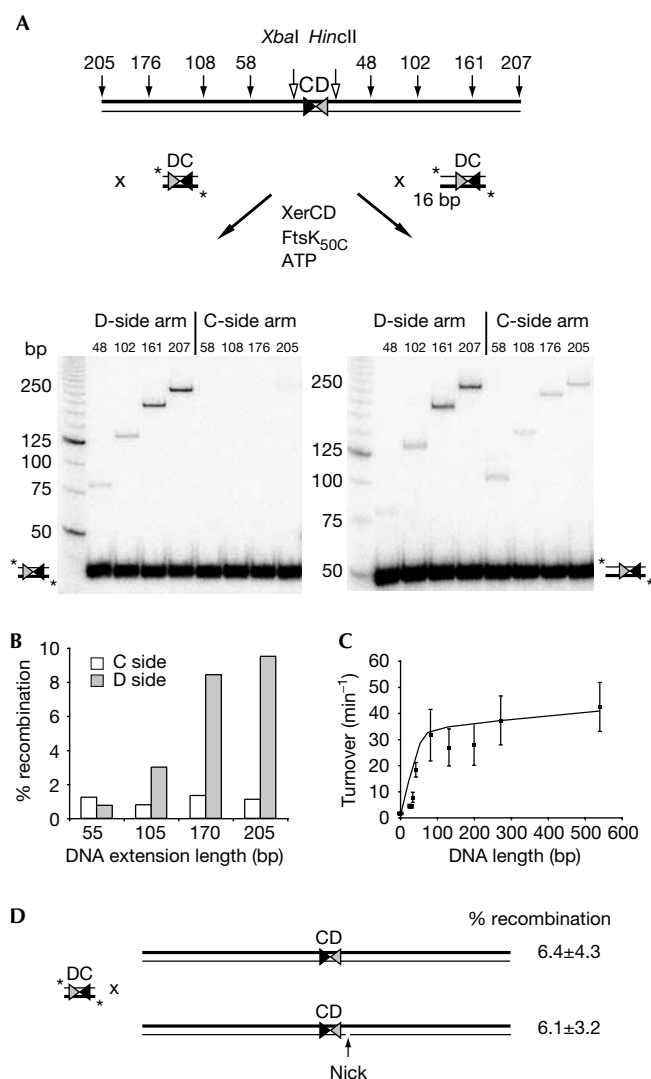

A

	Expected recombination products (bp)
*Mlu*I + *Nde*I fragment (M/N)	38* + 272*
*Sap*I + *Bgl*II fragment (S/B)	33* + 302*

Fig 3 | Asymmetric DNA requirement of FtsK$_{50C}$ is independent of DNA sequence. (**A**) Orientation of *dif* sites in pFX315 and pFX316, and the recombination products expected from reactions between *dif*-containing restriction fragments and a short, labelled *dif*-containing DNA (3CD3). Lowercase letters indicate DNA orientation. The single RAG sequence element is annotated. (**B**) Electrophoretic analysis of 60 min reactions detailed in (**A**). (**C**) Recombination over time for reactions between short, *dif*-containing DNAs where either one or both short fragments have a D-side extension. The reactions with just one D-side arm are between 3CD3 and 315 M/N.

Fig 5 | FtsK$_{50C}$ does not affect the direction or efficiency of resolution of a *dif*-containing HJ. The HJ schematized was prepared by annealing four oligonucleotides and then reacted with XerCD, in the presence or absence of FtsK$_{50C}$ and ATP, for 60 min at 37 °C. Reactions were deproteinized before 6% TBE–PAGE.

need to be transmitted through an uninterrupted phosphodiester backbone.

FtsK$_{50C}$ functions before HJ formation

Previous work showed that FtsK$_{50C}$ activates Xer recombination by mediating a switch in the catalytic state of the (XerCD–*dif*)$_2$ complex, so that recombination is initiated by XerD rather than by XerC (Aussel *et al*, 2002). Nevertheless, it remained unclear as to whether FtsK$_{50C}$ was only stimulating the first-strand exchange or whether it could also direct HJ resolution. We constructed a *dif*-containing HJ and analysed the effect of FtsK$_{50C}$ on its resolution by XerCD (Fig 5). In the absence of FtsK$_{50C}$, the HJ is resolved primarily by XerC-mediated cleavage into two duplex products. The presence of FtsK$_{50C}$, with or without ATP, has little effect on the HJ resolution reaction. The small increase in the level of XerD cleavage product in the presence of FtsK$_{50C}$ and ATP reflects intermolecular recombination between the two duplex products of XerC-mediated HJ cleavage. Therefore, FtsK$_{50C}$ appears not to modulate Xer recombination on an HJ substrate. Instead it acts only to promote the initial strand exchanges by XerD, generating an HJ intermediate that can spontaneously undergo XerC strand exchanges to form products in a reaction independent of FtsK$_{50C}$. This confirms the view obtained from studies of plasmid resolution (Aussel *et al*, 2002).

DISCUSSION

We have shown that FtsK$_{50C}$ can activate intermolecular Xer recombination between two short, linear *dif*-containing DNA fragments. In this ATP hydrolysis-dependent reaction, there is no room on the DNA arms for significant translocation by FtsK$_{50C}$, suggesting that the enzyme can use ATP hydrolysis to activate XerCD recombination directly. The observation that nicks on the DNA substrate, even as close as 3 bp from the *dif* site, do not prevent recombination activation by FtsK$_{50C}$ implies that changes in DNA torsion induced by FtsK$_{50C}$ are not essential to the activation mechanism. Furthermore, the fact that recombination activation by FtsK$_{50C}$ requires only one DNA arm adjoining an XerD-binding site in the (XerCD–*dif*)$_2$ synaptic complex suggests

Fig 4 | Effects of DNA arm length and backbone continuity on FtsK$_{50C}$ activities. (**A**) Unlabelled DNAs with different lengths of arm adjoining either the XerC side or the XerD side of *dif* were reacted with short, labelled *dif*-containing DNAs with either 3-bp arms (left panel) or 3 bp on the XerC side and 16 bp on the XerD side (3CD16; right panel), in the presence of XerCD, FtsK$_{50C}$ and ATP for 60 min. (**B**) Histogram comparing the levels of recombination observed when unlabelled DNAs with XerC side (white bars) or XerD side (grey bars) extensions were reacted with labelled 3CD16. The results are the means of three experiments. (**C**) Graph of DNA length against FtsK$_{50C}$ ATPase activity (mean turnover ± s.d. from three independent reactions, in the absence of XerCD). (**D**) A single-strand nick does not affect FtsK$_{50C}$ activation of recombination. Unlabelled DNA without (top reaction) or with (bottom reaction) a single-stranded nick 3 bp along the XerD DNA arm was reacted with labelled 3CD3 in the presence of XerCD, FtsK$_{50C}$ and ATP for 60 min. The results are the mean ± s.d. of three independent experiments.

activation step. Even a nick just three nucleotides away from the XerD-binding site of *dif* did not compromise activation of Xer recombination by FtsK$_{50C}$ (Fig 4D), thereby showing that activation is not dependent on torsional changes that
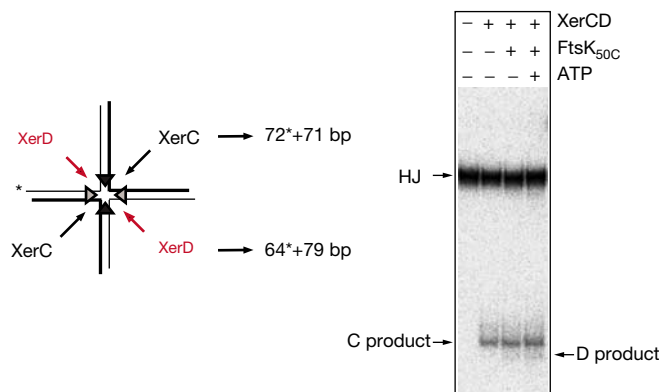
that FtsK$_C$ must interact with (XerCD–*dif*)$_2$ in a precisely oriented manner, possibly by directly contacting one XerD molecule. Such a model fits with the observation that XerD initiates strand exchange at *dif*, in contrast to FtsK-independent Xer reactions where XerC initiates recombination (Blakely *et al*, 2000; Aussel *et al*, 2002; Bregu *et al*, 2002). If the (XerCD–*dif*)$_2$ complex adopts a structure similar to that of the well-characterized tyrosine recombinase–DNA complex Cre-*loxP* (Guo *et al*, 1997, 1999), then switching on XerD catalysis and switching off XerC catalysis within (XerCD–*dif*)$_2$ must involve substantial reorganization of the macromolecular complex by FtsK$_C$, a process presumably driven by ATP hydrolysis. However, we cannot yet pinpoint the exact role of ATP hydrolysis by FtsK in the reaction mechanism: it may even be involved at a late stage to release the motor from the recombinase complex.

It is intriguing to note that the length of chromosomal DNA from the origin to the XerD half-site of *dif* is 29 kb (approximately 30 s replication time) shorter than the length from the origin to the XerC half-site of *dif*. If both replication forks moved at the same rate, the replicated *dif* sites would tend to be oriented such that the XerD half-sites are more distal to the moving fork than the XerC half-sites. This orientation might make the XerD arms more accessible to a translocase such as FtsK and could explain the evolution of an activatory FtsK–XerD interaction.

FtsK has been implicated in sensing chromosome polarity to set up productive translocation and *dif* synapsis (Pérals *et al*, 2000; Corre and Louarn, 2002). It has been hypothesized that chromosome polarity is defined by RAG elements, eight nucleotide DNA sequences with a highly skewed chromosomal distribution inverting at *dif* (Capiaux *et al*, 2001; Lobry & Louarn, 2003). In the experiments reported here, FtsK$_{50C}$ action was not influenced by the presence or absence of RAGs in either orientation on either arm of the unlabelled DNA (Fig 3A and data not shown). Although our data do not preclude a role for RAGs in global translocation, they clearly show that RAGs do not affect the local action of FtsK, and that although the FtsK$_C$ motor may contribute to *dif* synapsis *in vivo*, it must also be able to activate nucleoprotein complexes that have formed by random collision.

The assay developed here will facilitate further dissection of the molecular interactions involved in the chromosome dimer resolution reaction and should lead to a detailed view of how a radially symmetrical ring-shaped motor protein can lock onto and then actively remodel a protein–DNA complex to make that complex competent for recombination.

METHODS

Intermolecular recombination assays. Plasmids pFX315 and pFX316 were constructed by inserting wild-type *dif* into a derivative of pNB0 (Morgan *et al*, 2000). Short unlabelled *dif*-containing DNA fragments of variable length were produced by PCR on pMIN33 or by restriction digestion of pFX315 or pFX316. *N.BstNBI* (NEB) was used to create a single nick 3 bp away from *dif* adjacent to either the XerC-binding site (pFX316) or the XerD-binding site (pFX315). Short, ^{32}P-labelled, *dif*-containing DNA fragments were produced by annealing 5′-end-labelled oligonucleotides and purifying duplex DNA by 6% Tris–borate–EDTA polyacrylamide gel electrophoresis (TBE–PAGE). The short DNAs were eluted, ethanol-precipitated and resuspended in 50–200 µl TE (10 mM Tris–Cl, pH 8.0, 1 mM EDTA). XerCD and

FtsK$_{50C}$ proteins were purified as described previously (Aussel *et al*, 2002).

Intermolecular recombinations were carried out in 10 µl of reaction buffer (10 mM Tris–Cl, pH 7.5, 1 mM DTT, 10 mM MgCl$_2$, 1 mg/ml BSA, 40 mM NaCl) and contained approximately 2 nM labelled DNA, 2 nM unlabelled DNA, 150 nM XerC, 75 nM XerD, 300 nM FtsK$_{50C}$ (monomer concentrations) and 2.5 mM ATP or ATP-γS. FtsK$_{50C}$ was added last, to start the reaction. Reactions were incubated at 37 °C for up to 60 min before being stopped, deproteinized and analysed by 7% native TBE–PAGE. Gels were imaged and quantified using a Fuji PhosphorImager and ImageQuant software.

ATPase assays. ATP hydrolysis assays were carried out by thin-layer chromatography (TLC). Reaction mixtures (20 µl) containing 20 mM Tris–Cl, pH 7.5, 1 mM DTT, 40 mM NaCl, 10 mM MgCl$_2$, 50 ng DNA, 2 mM ATP and 1 nM [α^{32}-P] ATP were pre-incubated at 37 °C for 5 min before FtsK$_{50C}$ addition (150 nM). Reactions were incubated for a further 30 min before being stopped with EDTA (0.5 M, 2.5 µl), kept at 20 °C for 10 min and analysed by TLC on PEI–cellulose plates (Merck). ATP hydrolysis was directly proportional to time for at least 30 min, enabling enzyme turnover to be calculated in the presence of different DNAs.

HJ resolution. *dif*-containing HJs were prepared by annealing oligonucleotides (used to prepare untethered HJ in Arciszewska *et al*, 1997). HJs were purified twice by TBE–PAGE and ethanol precipitation before being used in resolution reactions at 2 nM. Reactions were performed using the same buffer and reactant concentrations as the intermolecular recombination reactions. Reactants were mixed on ice, and then FtsK$_{50C}$ was added immediately to some reactions. Reactions were incubated at 37 °C for 60 min before being stopped and analysed in the same way as the intermolecular recombination reactions.

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