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Asymmetric DNA requirements in Xer recombination activation by FtsK

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ABSTRACT

In bacteria with circular chromosomes, homologous recombination events can lead to the formation of chromosome dimers. In *Escherichia coli*, chromosome dimers are resolved by the addition of a crossover by two tyrosine recombinases, XerC and XerD, at a specific site on the chromosome, *dif*. Recombination depends on a direct contact between XerD and a cell division protein, FtsK, which functions as a hexameric double stranded DNA translocase. Here, we have investigated how the structure and composition of DNA interferes with Xer recombination activation by FtsK. XerC and XerD each cleave a specific strand on *dif*, the top and bottom strand, respectively. We found that the integrity and nature of eight bottom-strand nucleotides and three top-strand nucleotides immediately adjacent to the XerD-binding site of *dif* are crucial for recombination. These nucleotides are probably not implicated in FtsK translocation since FtsK could translocate on single stranded DNA in both the 5′–3′ and 3′–5′ orientation along a few nucleotides. We propose that they are required to stabilize FtsK in the vicinity of *dif* for recombination to occur because the FtsK–XerD interaction is too transient or too weak in itself to allow for XerD catalysis.

INTRODUCTION

Most bacteria harbour circular chromosomes. As a consequence, odd numbers of crossovers due to homologous recombination lead to the formation of chromosome dimers (1). Chromosome dimers threaten the segregation of genetic information if they are not taken care of at the time of cell division. In *Escherichia coli*, chromosome dimer resolution depends on the addition of a crossover at *dif*, a 28-bp site located at the opposite of the origin of replication, by two related tyrosine recombinases, XerC and XerD (2). This reaction can also serve to remove catenation links between newly replicated chromosomes when the activity of TopoIV, the major cellular decatenase, is compromised (3).

Recombination occurs within a nucleoprotein complex, $(XerCD–dif)_2$, which contains two molecules of each of XerC and XerD and two synapsed *dif* sites. The XerC and XerD recombinases sequentially catalyse two pairs of DNA strand exchanges within this complex, such that the reaction proceeds via a Holliday Junction (HJ) intermediate. Xer recombination is under the control of a cell division protein, FtsK (4), which permits its coordination with the last stage of cell division (5). FtsK can be divided into three domains (Figure 1A): an approximately 200 amino acids N-terminal domain ($FtsK_N$), a long linker region rich in proline and glutamine and an approximately 500 amino acids C-terminal domain ($FtsK_C$) (6,7). $FtsK_N$ contains four transmembrane helices, targets the protein to the division septum and is essential for cell division (8). $FtsK_C$ is an ATP-dependent DNA translocase (9,10). The *E. coli* chromosome carries preferential loading sites for $FtsK_C$, the KOPS (11,12). KOPS interact with the extreme C-terminal residues of FtsK, FtsKγ (13–15). They promote the loading of $FtsK_C$ in a precise orientation, which dictates the direction of translocation (11). KOPS are skewed on the two replichores of the *E. coli* chromosome: they point towards *dif* (12,16). This ensures that FtsK translocation brings together *dif* sites carried by a chromosome dimer. FtsK then activates recombination via a direct interaction between FtsKγ and the C-terminal tail of XerD (17,18).

Very little is known about the mechanism of DNA translocation by FtsK. $FtsK_C$ contains a RecA-type ATPase fold. In the presence of DNA, this fold assembles in hexameric ring structures, which encircle the DNA double helix (19). These structures convert the energy

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released by ATP hydrolysis into mechanical work to track along DNA at a velocity of up to 7 kb/s (10,20). Translocation induces very little rotation of $FtsK_C$ on the DNA, indicating that it does not track along the minor or major grooves of DNA (21). This raised questions on the DNA contacts it makes during translocation. Indeed, $FtsK_C$ is structurally related to hexameric helicases of the DnaB superfamily, which track along just one strand of DNA even when they move along duplex DNA (22). Similarly, motion of the EcoR124I double-stranded DNA translocase mainly depends on contacts to a single DNA strand, contacts to the other strand playing a role in stabilizing the motor on the DNA (23).

The mechanism of activation of Xer recombination by FtsK is also still enigmatic. XerC and XerD each cleave a specific strand on *dif*. By convention, these strands are termed the top and bottom strand, respectively. In the absence of FtsK, the (XerCD–*dif*)$_2$ complex can adopt a conformation suitable for XerC to mediate a first pair of strand exchanges (24). However, this pathway of recombination is a dead end, the HJ intermediate being rapidly converted back to substrate by another round of XerC catalysis (9,24). In the presence of FtsK, the (XerCD–*dif*)$_2$ complex adopts a conformation that allows XerD to mediate a first pair of strand exchanges, leading to a HJ that is resolved into a crossover by XerC catalysis (9). The switch in the catalytic properties of the (XerCD–*dif*)$_2$ complex depends on a direct contact between FtsKγ and XerD (17,18). However, FtsKγ alone does not promote Xer recombination between intact *dif* sites (18) and ATP hydrolysis is still required for recombination when $FtsK_C$ loads in the vicinity of *dif* (14,25), indicating a possible role for the RecA-type ATPase fold of $FtsK_C$ in recombination activation.

Here, we have investigated how the structure and composition of DNA interferes with the activity of FtsK using recombination reactions between short *dif*-containing DNA fragments, on which FtsK cannot load, and longer asymmetric DNA fragments, which allow the loading of FtsK on the XerD-side of *dif*. Promotion of recombination between these fragments requires that FtsK translocates along the longer fragment to contact the (XerCD–*dif*)$_2$ complex. More precisely, FtsK must track along the top strand of this fragment in the 3′–5′ orientation and along the bottom strand in the 5′–3′ orientation. We observed an asymmetric requirement for the integrity of top and bottom strands, the integrity of the bottom strand seeming to prevail for efficient recombination. However, the effect of DNA modifications decreased as a function of their distance to *dif*, suggesting a local inhibition of the recombination process rather than an inhibition of FtsK translocation. Indeed, we found that FtsK can track for a few nucleotides along a single DNA strand in both 5′–3′ and 3′–5′ orientations, as judged by its capacity to displace short oligonucleotides bound to single-stranded DNA. Based on these results, we propose that the FtsKγ–XerD interaction is too transient or too weak in itself to allow for XerD catalysis and that specific DNA contacts are required to stabilize FstKγ in the vicinity of (XerCD–*dif*)$_2$ for recombination to occur.

MATERIALS AND METHODS

DNA substrates and proteins

Recombination and probe displacement substrates were obtained by annealing gel-purified synthetic oligonucleotides, which were purchased from Eurogentec or Sigma (Supplementary Table 1). *Escherichia coli* $FtsK_{50C}$ was purified as a C-terminal fusion to GST, 6His and the FLAG epitope (GST–6His–FLAG–$FtsK_{50C}$). *Escherichia coli* XerC and XerD were purified as a C-terminal fusion to 6His (6his–XerC of 6his–XerD). Those N-terminal tags did not interfere with the activities of the proteins, as indicated by the high efficiency of recombination obtained in our *in vitro* assays (Figure 1C). Proteins were expressed from pET derivative plasmids (GST–$FtsK_{50C}$ in pLB14, 6His–XerC in pFX131 and 6His–XerD in pFX134) transformed in *E. coli* B834 cells. GST–$FtsK_{50C}$ was produced at 37°C for 3 h. 6His–XerC and -XerD were produced at 30°C for 1 h. Recombinant proteins were purified on an AKTA Purifier (GE Healthcare) using a Histrap column as a first step. As a second step, we used a heparin column or a GSTrap column for the purification of XerCD or $FtsK_{50C}$, respectively. GST–$FtsK_{50C}$ was stored in a buffer containing 15% glycerol, 40 mM Tris–HCl pH 8, 150 mM NaCl, 10 mM of reduced glutathione, 1 mM dithiothreitol (DTT) and 1 mM ethylenediaminetetraacetic acid (EDTA) at −80°C. 6His–XerC and -XerD were stored in a buffer containing 40% glycerol, 50 mM Tris–HCl, pH 8, 1 M NaCl, 1 mM DTT and 1 mM EDTA at −80°C.

Xer recombination assay

Recombination assays were performed as described previously (12,25). The short *dif*-containing probes were labelled at the 5′-end of the strand cleaved by XerC (top strand) using T4 DNA polynucleotide kinase and [©-32P]ATP and purified using a MicroSpin™ G-25 column (GE Healthcare). Intermolecular recombinations were carried out in 10 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 0.1 mg/ml BSA and 10 mM NaCl, in a volume of 10 µl. Reactions contained 2 nM of labelled probe DNA, 2 nM of unlabelled DNA, 150 nM of XerC, 75 nM of XerD, 300 nM of $FtsK_{50C}$ (monomer concentration) and 2.5 mM of ATP. ATP was added last, to start the reactions. Reactions were incubated at 37°C for 30 min and stopped by the addition of 2 µl of a 30% glycerol, 3% SDS, 2.5 µg/ml proteinase K, 100 mM EDTA and 50 mM Tris–HCl, pH 8 solution. Reaction products were separated by 7% TBE–PAGE and quantified using a Storm PhosphorImager (Molecular Dynamics) and ImageGauge software.

Gel mobility shift assay

Binding reactions were performed at 37°C for 10 min in the same buffer and proteins concentrations as recombination reaction but in the absence of FtsK and ATP. Two microlitres of 40% glycerol were added to the samples prior to loading on 7% TBE–PAGE. Gels were run for 4 h at 4°C. Gels were imaged and quantified as described above.

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Probe displacement assay

Reactions were carried out in 25 mM Tris–Acetate pH 7.9, 0.2 mM DTT, 10 mM $MgCl_2$ and 0.1 mg/ml casein in a total volume of 10 µl. Reactions contained 2 nM of labelled DNA, 300 nM of $FtsK_{50C}$ (monomer concentration) and 2.5 mM of ATP. Reactions were incubated at 37°C for only 5 min to limit the amount of oligonucleotides that are passively liberated. Reactions were stopped with 2 µl of a 30% glycerol, 1.25% SDS, 10 mM Tris–HCl pH 7.5, 0.06% bromophenol bromide and 0.06% xylene cyanol solution. Reaction products were analysed by 7% TBE–PAGE and quantified as described above.

RESULTS

Xer recombination activation on short asymmetric substrates

Complete recombination reactions between *dif* sites can be reconstituted *in vitro* using a purified truncated version of FtsK, $FtsK_{50C}$, which retains both translocation and Xer recombination activities (9,10). Four duplex DNA segments extend from the (XerCD–*dif*)$_2$ recombination complex. However, loading of $FtsK_{50C}$ on a single duplex DNA fragment is sufficient for recombination *in vitro*, provided that this fragment extends from the XerD side of the complex (25). In such reactions, DNA modifications positioned between *dif* and the loading site of $FtsK_{50C}$ could affect the efficiency of recombination in two ways. First, they could stop $FtsK_{50C}$ translocation, thereby impeding FtsKγ from contacting XerD (Figure 1B, i). Such an indirect effect has been previously observed with KOPS inserted in the non-permissive orientation in the vicinity of *dif* (12). Second, they could impede any active role that the ATPase engine of $FtsK_{50C}$ might have in the recombination process (Figure 1B, ii), as suggested by the requirement for ATP hydrolysis in recombination reactions among intact *dif* sites (14,18,25). Recombination reactions on short asymmetric substrates therefore provide a very sensitive assay to test the impact of DNA modifications on the activity of the $FtsK_{50C}$ engine. Here, we used synthetic oligonucleotides to create a short DNA fragment carrying only 3 bp of duplex DNA on either side of *dif* (Figure 1C, 3CD3). This fragment was radiolabelled on the 5′-end of the top strand. It was used in recombination reactions against longer DNA fragments, which were also created by hybridization of synthetic oligonucleotides and which carried 3 bp of duplex DNA on the XerC side of *dif* and 89 or 109 bp on the XerD side (Figure 1C, 3CD89 and 3CD109). A triple KOPS motif (11) was inserted at 35 bp from the extremity of those long DNA fragments to ensure efficient loading of FtsK (Figure 1B, 3KOPS). When <114 bp of duplex DNA are adjacent to the XerD side of *dif*, the efficiency of recombination is over 10 times more efficient in the presence of the triple KOPS motif than in its absence (11,25), suggesting that FtsK does not load efficiently on DNA molecules smaller than 114 bp (25). We decided therefore to use short 89- and 109-bp DNA extensions on the XerD side of *dif* to further limit random FtsK loading at other places than the

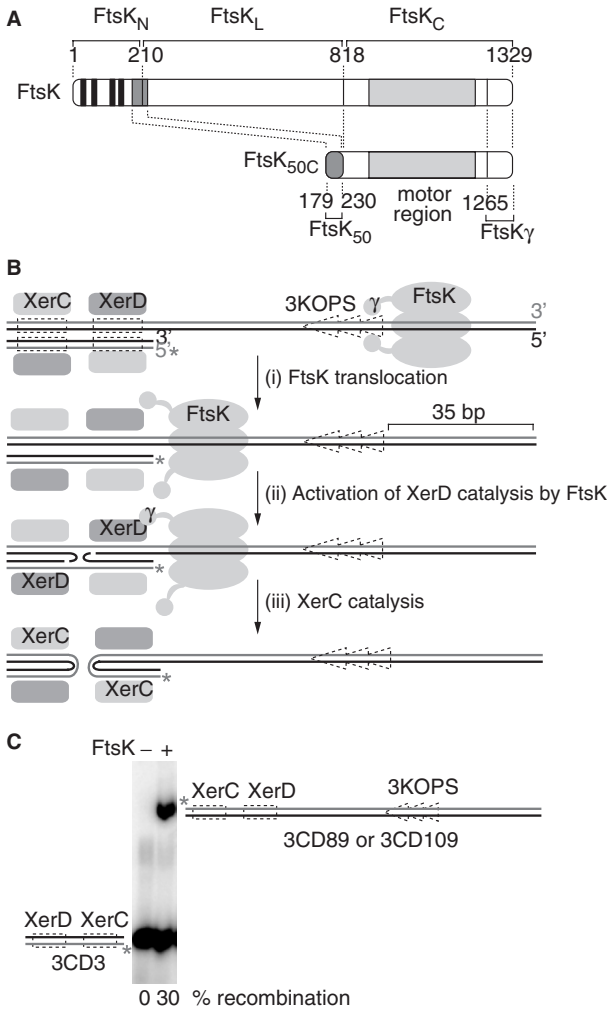


Figure 1. Asymmetric Xer recombination assay. (**A**) Schematic of *E. coli* FtsK. The positions of the amino acid residues delimiting the different domains of the protein are indicated. Black bars depict the transmembrane domains. (**B**) Recombination between a long substrate containing a triple KOPS motif (3KOPS) oriented towards *dif* and a short radiolabelled *dif* probe. Top and bottom strands are depicted as grey and black lines, respectively. The 5′ and 3′ indicate the polarity of the strands. The radioactive label on the short substrate is indicated by a star. FtsK loads on the KOPS, translocates (i) and activates catalysis by the XerD recombinases (ii), leading to the formation of Holiday junction. The XerD recombinases cleave the bottom strands. The XerC recombinases then cleave the top strands (iii). The resulting products are a short unlabelled probe and a long radiolabelled fragment. (**C**) Recombination reaction between 3CD3 and 3CD89.

3KOPS. $FtsK_{50C}$ promoted a very high level of recombination between equimolar amounts of 3CD3 and 3CD89 or 3CD109 (Figure 1C).

Top- and bottom-strands asymmetry

We first tested if $FtsK_{50C}$ could promote Xer recombination when a single stranded gap was present between *dif* and the triple KOPS motif on 3CD89 (Figure 2A). Five base pairs of duplex DNA were left adjacent to *dif*, to avoid any interference in the way XerC and XerD bind and cleave *dif* (25). The gaps were positioned either

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on the top or the bottom strand of the 3CD89 substrates, which are, respectively, cleaved by XerC and XerD (Figure 2A). Note that $FtsK_{50C}$ must track the top strand in the 3′–5′ orientation and the bottom strand in the 5′–3′ orientation to reach *dif* when it has loaded at the triple KOPS (Figure 2A). On either the top strand or the bottom strand of *dif*, 5, 10 and 30 nt gaps severely impaired Xer recombination (Figure 2B). Efficient recombination was recovered when gaps were filled with DNA, leaving two nicks between non-phosphorylated nucleotides at 5 and 35 bp from *dif* (Figure 2B, Nicks). Thus, the continuity of the top and bottom strands is not important for FtsK activity, as previously observed with a single nick at 5 bp from *dif* (25). However, duplex DNA is required between *dif* and the loading site of FtsK for recombination to occur. Finally, we noted that recombination efficiency was much more affected when gaps were on the bottom strand than on the top strand of *dif* (Figure 2C).

Top- and bottom-strands asymmetry is not sequence-specific

The existence of KOPS illustrates how the sequence composition of a DNA fragment can modify the activity of $FtsK_{50C}$ (11,12,16). It was therefore possible that the top- and bottom-strands asymmetry observed in Figure 2 was due to the orientation and/or composition of the DNA sequence separating *dif* and the KOPS in 3CD89. This hypothesis was tested using a substrate in which the sequence between *dif* and the KOPS was inverted (Figure 3A, i3CD89). Introduction of nicks or of 5, 10 and 30 nt gaps in the top and bottom strands of i3CD89 yielded similar results than those obtained with 3CD89 (Figure 3B and C), indicating that the base composition of DNA does not influence recombination activation.

Importance of the sugar–phosphate backbone of the bottom strand

The above results suggested that recombination activation did not depend on contacts between FtsK and the bases of DNA, but rather on contacts between FtsK and the sugar–phosphate backbone of DNA. This was tested by filling 30 nt top-strand or bottom-strand gaps with RNA (Figure 3D, RNA). Filling the 30 nt top-strand gaps with RNA restored recombination (Figure 3E, RNA, Top). But filling bottom-strand gaps did not restore recombination (Figure 3E, RNA, Bot). To check for the integrity and the capacity of the synthetic RNA oligonucleotides to anneal to the gaps, we performed those experiments with two different RNA oligonucleotides, RNA1 and RNA2: RNA1 served to fill the 30 nt top-strand gap on 3CD89 and the bottom-strand gap on i3CD89; RNA2 served to fill the 30 nt top-strand gap on i3CD89 and the bottom-strand gap on 3CD89 (Figure 3D). There are two major differences between RNA and DNA: the presence of a hydroxyl group in position 2′ of the sugar–phosphate backbone of RNA and the replacement of thymine by uracil (Figure 3D). Filling the 30 nt bottom-strand gap with DNA containing uracil bases instead of thymine fully restored recombination activation (Figure 3E, dU).

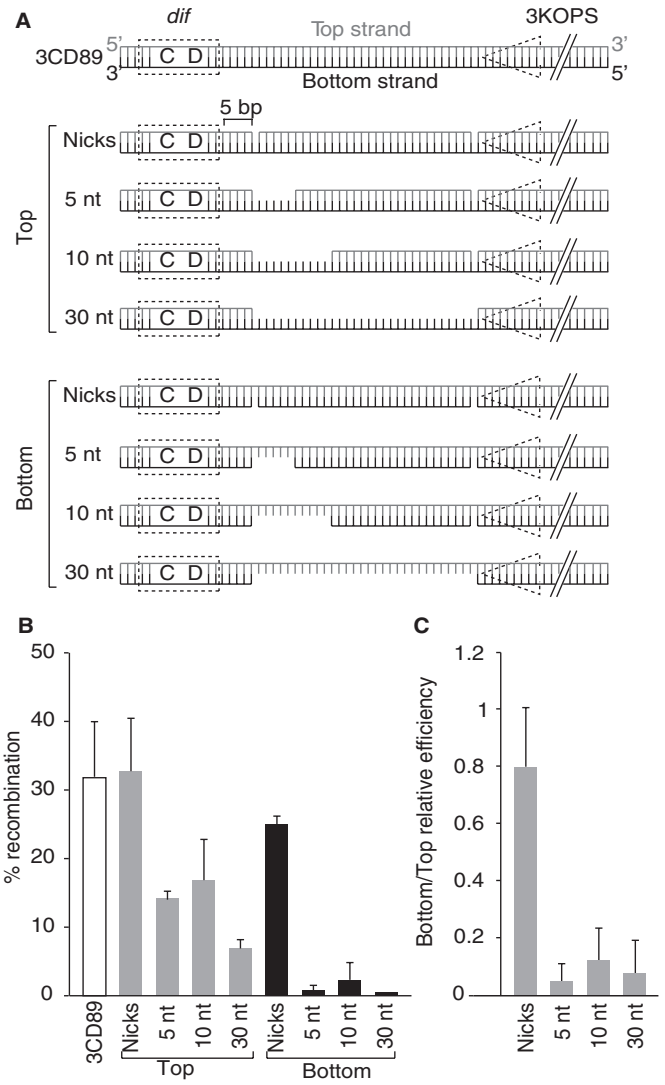


Figure 2. Top- and bottom-strand gaps affect recombination efficiency. (**A**) Scheme of 3CD89 recombination substrates with nicks or gaps of different length on the top or bottom strand. No phosphate residues are left at the position of nicks. (**B**) Efficiency of recombination for the different 3CD89 substrates (mean and standard deviations of four independent experiments). (**C**) Relative efficiency of recombination of substrates with bottom-strand gaps versus substrates with top-strand gaps (mean and standard deviations of four independent experiments).

Thus, the integrity and the nature of the sugar–phosphate backbone of the bottom strand of the substrate determine the efficiency of recombination.

A 2 nt gap on the bottom strand of *dif* blocks Xer activation

Since 5, 10 and 30 nt bottom-strand gaps positioned at 5 bp from *dif* yielded the same dramatic decrease in Xer recombination activation (Figures 2 and 3), we investigated if smaller gaps could also affect FtsK-dependent Xer recombination (Figure 4A). On the top strand, the efficiency of recombination diminished when the size of the gaps was increased (Figure 4B). However, recombination was not totally abolished, even with a 30 nt gap. In contrast, the efficiency of recombination dropped to the

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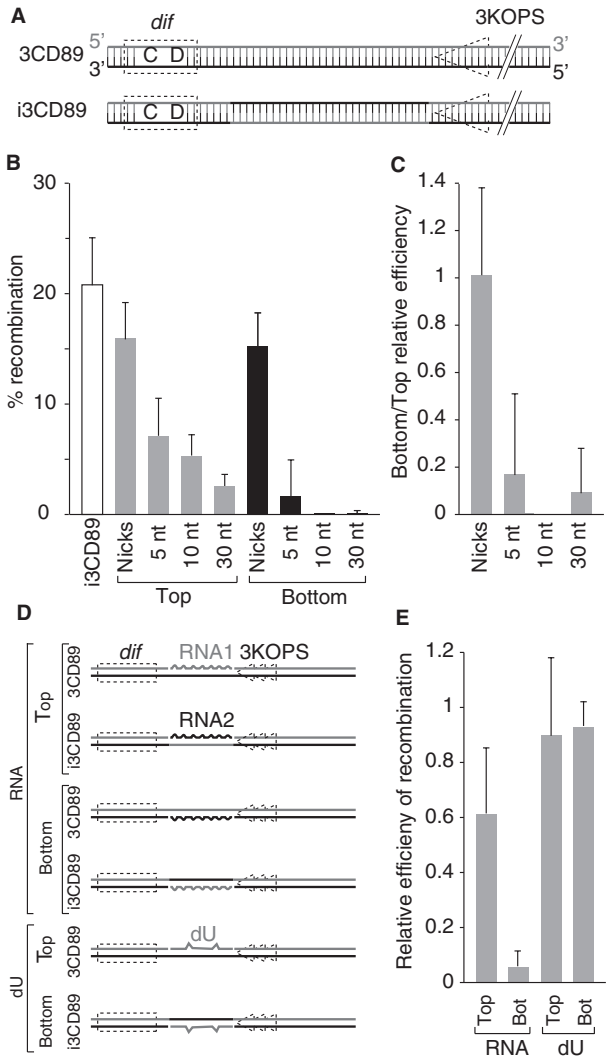


Figure 3. Top- and bottom-strands asymmetry is not linked to base composition. (**A**) Scheme of i3CD89 recombination substrates, which present a 30-bp inverted region between *dif* and the 3KOPS motif compared to 3CD89 substrates. (**B**) Efficiency of recombination for the different i3CD89 substrates (mean and standard deviations of four independent experiments). (**C**) Relative efficiency of recombination of i3CD89 substrates with bottom-strand gaps versus substrates with top-strand gaps (mean and standard deviations of four independent experiments). (**D**) Scheme of 3CD89 and i3CD89 recombination substrates with 30 nt gaps on top or bottom strand filled with RNA or DNA containing deoxyuridins (dU). (**E**) Relative efficiency of recombination of RNA and dU substrates (the number of independent experiments are indicated upon each data column). No differences were observed between 3CD89 and i3CD89 RNA substrates and results were pooled.

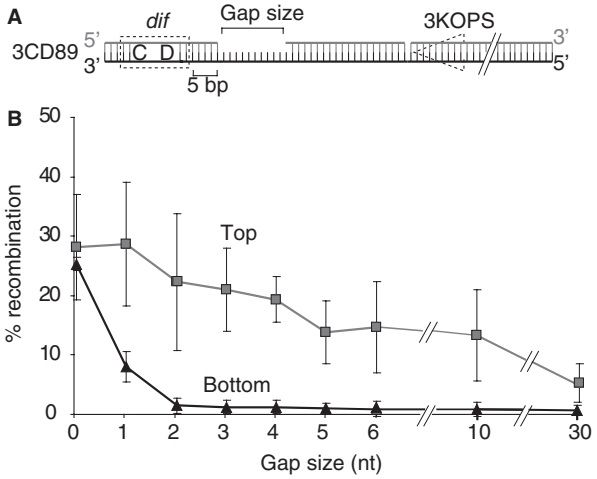


Figure 4. A 2 nt gap on the bottom strand of DNA stops recombination activation. Efficiency of recombination (**B**) using substrates with variable gap size at 5 nt from *dif* (**A**).

background level of the assay when gaps larger than 2 nt were created on the bottom strand of the recombination substrate (Figure 4B).

Probing for nucleotides implicated in Xer recombination activation

The dramatic effect of 2 nt gaps on the efficiency of FtsK-dependent Xer recombination suggested that it should be possible to precisely map which of the nucleotides in the immediate vicinity of *dif* are crucial for the activity of $FtsK_{50C}$ by monitoring recombination on substrates containing 2 nt gaps at various distance from *dif* (Figure 5A). On the top strand, the efficiency of recombination dropped to less than a third of its normal level when 2 nt gaps were positioned at 0 and 1 bp from *dif* (Figure 5B, Top). A 2 nt gap at 2 bp from *dif* yielded an intermediate level of recombination, while gaps further away from *dif* had no effect on the efficiency of recombination (Figure 5B, Top). On the bottom strand, the efficiency of recombination dropped to less than a third of its normal level for 2 nt gaps positioned at 1–7 bp from *dif* (Figure 5B, Bottom). Two nucleotides gaps at 0 or 8 bp from *dif* yielded intermediate levels of recombination, while gaps further away from *dif* had no effect on the efficiency of recombination (Figure 5B, Bottom). A similar profile was obtained with single base pair gaps, but the smaller inhibition of recombination did not allow as precise a mapping as with 2 nt gaps because of the experimental noise (data not shown). Finally, nicks on the top or bottom strands at positions ranging from 0 to 8 bp from *dif* (Figure 5C) had no significant effect on recombination at any position (Figure 5D). Thus, the presence of the 3 nt immediately adjacent to *dif* on the top strand is important for FtsK-dependent Xer recombination. On the bottom strand, the presence of the nucleotides ranging from position 2–9 from *dif* is important for FtsK-dependent Xer recombination.

The duplex DNA adjacent to *dif* is not required for XerCD binding and activity

Because the gaps that had the strongest effect on Xer recombination were those that are immediately adjacent to the XerD-binding site of *dif*, we wanted to check that their inhibitory effect was not linked to a decrease in the efficiency of binding of XerC and XerD to the substrate or to a decrease in their capacity to cleave and rejoin DNA. Under the same conditions as those in which we

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performed our recombination experiments, XerC and XerD were able to fully retard the migration of 3CDtop, which lacks the 3 nt immediately adjacent to the XerD-binding site on the top strand, 3CDbot, which lacks the 3 nt immediately adjacent to the XerD-binding site on the bottom strand, and 3CD0, which totally lacks duplex DNA on the XerD side (Figure 6A). In addition, equivalent levels of recombination between an intact 3CD89 fragment and 3CD3, 3CD0, 3CDtop and 3CDbot were obtained (Figure 6B). Thus, the absence of duplex DNA in the immediate vicinity of the XerD-binding site of *dif* does not inhibit the binding and the catalytic activities of XerC and XerD.

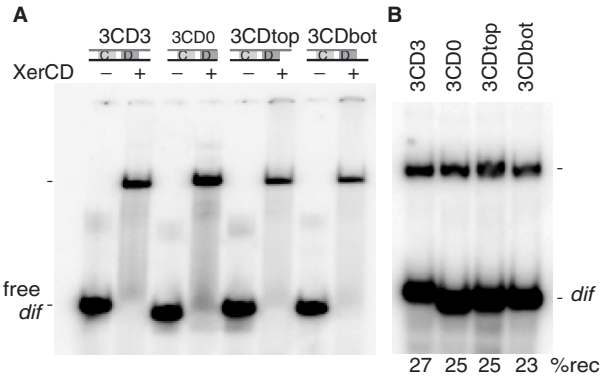


Figure 6. The duplex DNA adjacent to *dif* is not required for XerCD binding and activity. (**A**) Gel mobility shift assay showing equivalent binding of XerCD recombinases on short *dif* substrates: 3CD3 carries 3 bp on the XerD side of *dif*, 3CDtop and 3CDbot, respectively, carry 3 nt of single-stranded DNA on the top or bottom strands of DNA on the XerD side of *dif*, 3CD0 entirely lacks DNA on the XerD side of *dif*. (**B**) PAGE showing equivalent recombination efficiency between 3CD89 and 3CD3, 3CD0, 3CDtop or 3CDbot.

Positioning gaps away from *dif* attenuates top and bottom-strands asymmetry

Since the effect of 2 nt gaps was abolished at 9 bp from *dif* (Figure 5), we decided to check if the effect of longer gaps was also dependent on their distance to *dif*. To this aim, we used a longer recombination substrate,

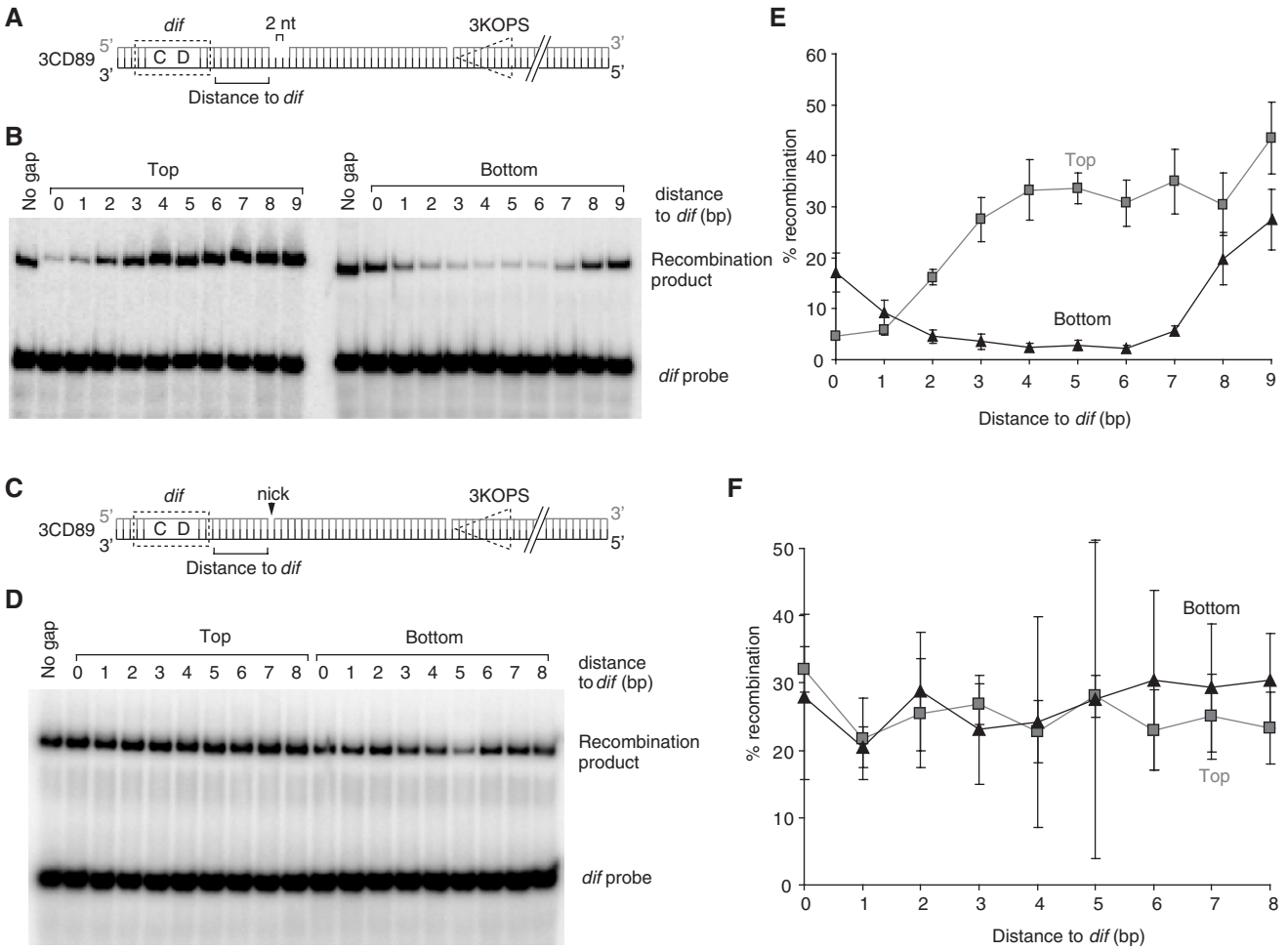


Figure 5. Scanning for important nucleotides in the immediate vicinity of *dif*. 3CD89 recombination substrates presenting 2 nt gaps (**A–C**) or nicks (**D–F**) at variable position from *dif*. Schemes of the substrates (A, C); gels displaying typical recombination results (B, D); mean efficiency of recombination from three independent experiments (E, F). The largest error bars in Figure 5F correspond to the substrates with nicks on the bottom strand.

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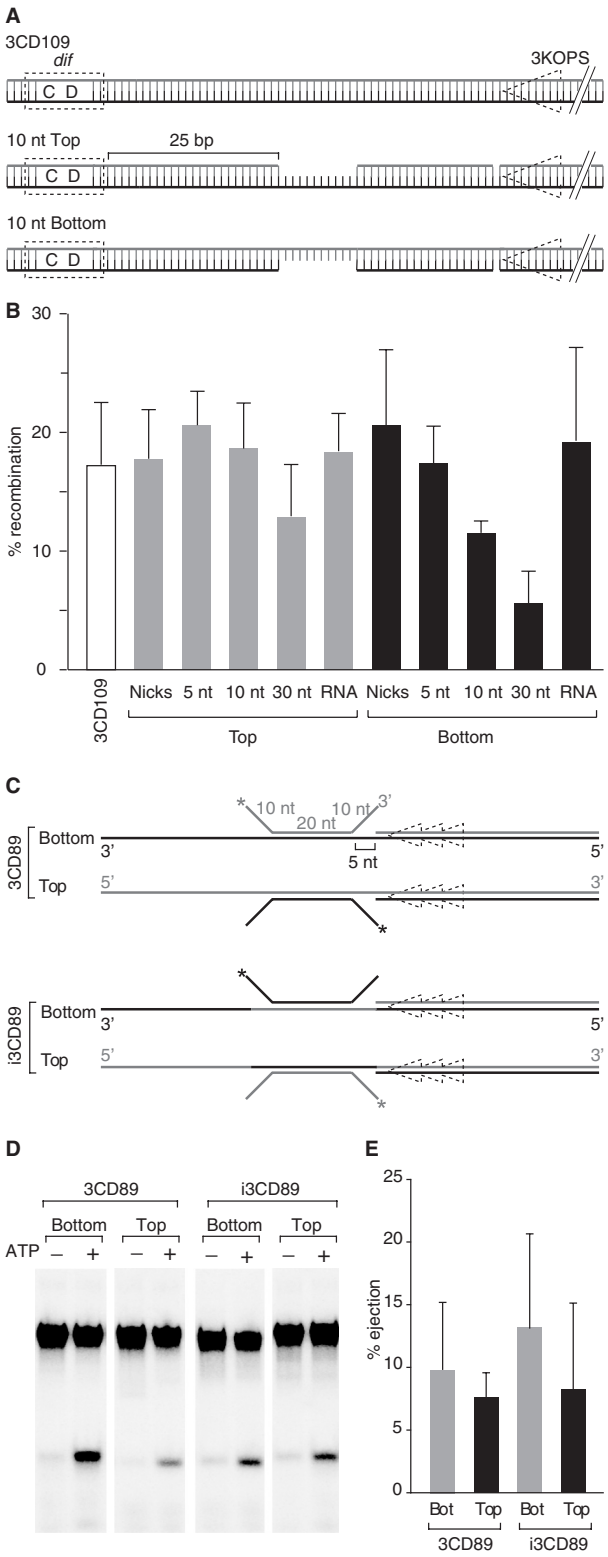


Figure 7. The effect of gaps is local. (**A**) Scheme of 3CD109 recombination substrates, which contain 20 more base pairs between *dif* and the 3KOPS motif than 3CD89 substrates. (**B**) Efficiency of recombination on 3CD109 substrates with gaps at 25 bp from *dif* (mean and standard deviations of three independent experiments). (**C**) Scheme of the oligonucleotide displacement substrates based on 3CD89. The loading site of FtsK (including the 3KOPS and the 35 bp tail) is double-stranded. Radiolabelled oligonucleotides are annealed to 20 bases of 3CD89 at 5 nt from the duplex DNA loading site of FtsK. Those oligonucleotides carry 10 nt flap extensions with no homologies to 3CD89. They are annealed to either the top or the bottom strand of 3CD89. Oligonucleotide displacement then requires FtsK to track along the 5 nt single-stranded DNA region between its duplex DNA loading site and the flap structures, either on the top or the bottom strand (Bot). The 5′ and 3′ indicate the polarity of the strands. (**D**) Gel presenting a typical flap ejection experiment and (**E**) mean and standard deviation of the efficiency of displacement measured in three independent experiments.

3CD109, in which 5, 10 and 30 nt gaps could be positioned at 25 bp from *dif* instead of 5 bp (Figure 7A). At 25 bp from *dif*, top-strand DNA modifications had no significant effect on the efficiency of recombination (Figure 7B, Top). Likewise, the presence of a 5 nt gap or of 30 nt of RNA at 25 bp from *dif* on the bottom strand did not alter the efficiency of recombination (Figure 7B, Bottom). Finally, 10 and 30 nt bottom stand gaps had a much less dramatic effect on the efficiency of recombination at 25 bp from *dif* (Figure 7B, Bottom) than at 5 bp from *dif* (Figures 2 and 3). However, recombination efficiency of 10 and 30 nt bottom-strand gaps positioned at 25 bp from *dif* was still lower than the one of intact or nicked substrates (Figure 7B, Bottom). We did not design longer substrates to test if the effect of 10 or 30 nt gaps could be abolished further away from *dif* because we would not be able to exclude that the recovery of recombination on such longer substrates might not be linked to the loading of FtsK on the intact duplex DNA situated between *dif* and the gap rather than on the ability of FtsK to translocate on single-stranded DNA.

$FtsK_{50C}$ can function as both a 5′–3′ and a 3′–5′ DNA helicase

We then decided to test the effect of gaps on the translocation activity of FtsK using T-assays, in which we monitor the capacity of $FtsK_{50C}$ to displace an oligonucleotide bound at the extremity of a DNA fork (12). While performing those experiments, we noticed that $FtsK_{50C}$ could displace an oligonucleotide bound to either the top or to the bottom strand of the forked structure (Supplementary Figure 1). In other terms, the $FtsK_{50C}$ DNA translocase can be converted into a DNA helicase. We observed a high efficiency of displacement when 20 bases of the oligonucleotide paired on the translocation substrate, but very little if pairing reached 34 or 40 bp (data not shown), indicating that $FtsK_{50C}$ poorly functions as a helicase and probably stalls during the process. Displacement depended on the presence of an unpaired oligonucleotide tail, or flap, of >10 nt (Supplementary Figure 1), further indicating that the helicase activity of FtsK is probably due to the obstruction of the channel through which DNA is pumped in the hexameric FtsK rings. Based on these observations, we designed two 40 nt oligonucleotides, with a 20 nt central region pairing with either the bottom strand or the top strand of the 30 nt gap region of 3CD89 and i3CD89 (Figure 7C). These oligonucleotides were bound at a distance of 5 nt from the end of the duplex DNA fragment on which $FtsK_{50C}$ was loaded (Figure 7C). $FtsK_{50C}$ could efficiently unwind both oligonucleotides on either of the two substrates (Figure 7D). In addition, we observed no significant differences

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between the efficiency of displacement, whether the oligonucleotides were annealed to the top or to the bottom strand (Figure 7E). Thus, FtsK seems to be able to translocate on single-stranded DNA for at least 5 nt in both the 5′–3′ and 3′–5′ orientation.

DISCUSSION

Identification of nucleotides implicated in the activity of FtsK

We have investigated the importance of the structure and composition of DNA on the activity of FtsK by monitoring Xer recombination between short, asymmetric linear DNA fragments carrying DNA modifications between the loading site of $FtsK_{50C}$ and *dif* (Figure 1). This assay was chosen because it is quantitative (12) and because it was expected to be very sensitive. We thus found that activation of Xer recombination is dramatically affected when single-stranded gaps are present on the bottom strand of *dif* (Figures 2 and 3). Bottom-strand gaps as short as 2 nt reduced recombination to background levels (Figure 4), which allowed us to scan for specific nucleotides in the vicinity of *dif* that would be implicated in the activation process (Figure 5). This analysis revealed that on the bottom strand, 8 nt are crucial for recombination, from the second to the ninth nucleotide immediately adjacent to the XerD-binding site of *dif*, and that the three top-strand nucleotides immediately adjacent to the XerD-binding site of *dif* are also crucial for recombination (Figure 8). Finally, we showed that XerC and XerD do not require those nucleotides for binding to *dif*, nor for cleaving and rejoining DNA (Figure 6).

Nature of the contacts between FtsK and DNA

What is important in the nucleotides we have identified? The nature of the bases positioned at >5 nt form *dif* is not implicated in the recombination process, since inverting the sequence of the DNA region on the XerD side of *dif* or replacing thymine bases by uracil bases did not affect recombination (Figure 3). In contrast, replacing part of the bottom strand by RNA severely inhibited recombination (Figure 3). The DNA double helix is generally in the B-conformation, whereas RNA/DNA hybrids adopt the A-conformation. Replacing 2 nt of the bottom strand by locked nucleic acids, which also adopt the A-conformation when paired with DNA, did not impede recombination, suggesting that the conformation of the double helix does not play a role in the activation process (data not shown). Indeed, the efficiency of recombination reactions in the presence of 2 nt top-strand gaps positioned at 4 bp from *dif* (Figure 4) or of mismatched bases (data not shown) further indicates that the base pairing and/or the conformation of the double helix are not important for recombination. Thus, recombination activation does not depend on contacts between FtsK and the bases of DNA or on the particular helical structure of DNA but rather on contacts between FtsK and the sugar–phosphate backbone of DNA.

Influence of the local DNA structure on Xer recombination activation

The effect of top and bottom-strand gaps on recombination was attenuated when they were displaced away from *dif* (Figures 5 and 7), indicating that they locally influence the recombination process. Activation of recombination by FtsK requires a direct contact between FtsKγ and XerD (17,18). This contact has to occur in *cis*, since FtsK cannot activate recombination when loaded on the XerC side of the complex on short asymmetric substrates, whether KOPS are present (data not shown) or not (25). FtsKγ is connected to the $FtsK_{50C}$ engine by a region of approximately 15 aa (including a large proportion of glycine residues), which is probably unstructured and could extend to 4 or 5 nm. This should be sufficient to allow FtsKγ to contact XerD in *cis*, even if the FtsK motor stalled 3 bp before the $(XerCD–dif)_2$ complex, as would be the case for 2 nt gaps positioned at 0 or 1 bp from *dif* (Figure 8). Thus, it is unlikely that 2 nt gaps in the immediate vicinity of *dif* impede the interaction of FtsKγ and XerD. It would be therefore tempting to propose that $FtsK_{50C}$ needs to change the conformation of the $(XerCD–dif)_2$ complex in addition to contacting XerD. The top and bottom-strands asymmetry could then be explained by the fact that some mechanical stress needs to be propagated through the bottom strand of the substrate, i.e. the strand cleaved by XerD, for this change of conformation to occur. Indeed, the minimum size of bottom-strand gaps abolishing Xer recombination is 2 nt (Figure 5), which fits remarkably well with the step size of FtsK monomers, as estimated from crystallographic data (19). Two types of mechanical stress could occur, torsion or flexion. Torsion is ruled out because (i) the motion of $FtsK_{50C}$ induces very little rotation relative to the DNA (21) and (ii) the presence of nicks has no effect on the efficiency of recombination (Figure 5). Flexion is also very unlikely since FtsK can translocate on single-stranded

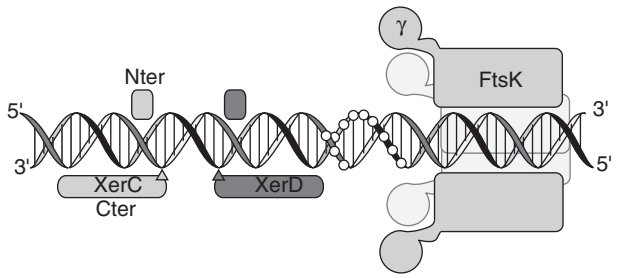


Figure 8. FtsK–DNA interactions during Xer recombination activation. The scheme represents a side view of the substrate on which FtsK is loaded. The top strand is shown as a light grey ribbon. The bottom strand is shown as a black ribbon. The 5′ and 3′ indicate the polarity of the strands. Base pairings are indicated by vertical bars. Light and dark grey triangles indicate the point of cleavage of XerC and XerD, respectively. The two foremost subunits of the FtsK complex have been left out, for clarity. White circles indicate top- and bottom-strand nucleotides implicated in Xer recombination activation by FtsK. The linker between the FtsK motor and FtsKγ could possibly reach 5 nm. However, in the absence of structural data, we decided to draw it at an intermediate size.

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DNA in both the 5′–3′ and 3′–5′ orientation along at least 5 nt, which should allow it to push on the (XerCD–*dif*)$_2$ synapse, even if a few nucleotides are missing in the vicinity of *dif* (Figure 7). In addition, we observed that XerC and XerD can stop the translocation of FtsK on DNA when bound to *dif* (Bonné,L. *et al.*, unpublished data), which should limit the amount of torsion and flexion that FtsK could introduce in the vicinity of the complex. Therefore, we propose that the specific nucleotides we identified in the immediate vicinity of *dif* (Figure 8) serve to stabilize FstK at the proximity of the (XerCD–*dif*)$_2$ complex, to allow for a sufficiently long and stable contact between FtsKγ and XerD for recombination to occur.

Top and bottom-strand asymmetry

The FtsK hexamer seems to make preferential contacts with the bottom strand of DNA since 8 nt of the bottom strand are important for Xer recombination activation versus only three on the top strand in the immediate vicinity of *dif* (Figure 5) and since only bottom-strand gaps affect recombination further away from *dif* (Figure 7). FtsK needs to track along the bottom strand in the 5′–3′ direction to activate recombination. This could indicate that the FtsK hexamer functions as a 5′–3′ translocase, like DnaB (22) and that DNA contacts with the top strand of DNA only serve to stabilize the motor on DNA, as suggested for EcoR124I (23). Indeed, most helicases or DNA translocases move on DNA with a preferential polarity (22). However, we did not observe any asymmetry between the top and bottom strands of DNA in oligonucleotide displacement assays (Figure 7), suggesting that FtsK can function as both a 5′–3′ and a 3′–5′ DNA helicase. Although surprising, this result was not entirely unexpected since a similar bipolar helicase activity has already been reported for HerA, a DNA helicase from archaea belonging to the FtsK family of translocases (26). However, we wish to emphasize that this does not exclude a possible effect of bottom-strand gaps on FtsK translocation. Indeed, the preferential loading of the EcoR124I motor on the 3′–5′ strand only led to a delay of a few seconds in triplex displacement assays when gaps were present in this strand, EcoR124I being able to bypass gaps of any size when given sufficient time in those assays (23). Complete stalling of EcoR124I by gaps on the 3′–5′ strand required a force to be applied on the DNA template on which it translocates using magnetic tweezers (23). Thus, further experiments will be required to determine if one of the two strands of the DNA helix is more important than the other for FtsK translocation.

The asymmetry between the top and the bottom strand of DNA might also be linked to the particular structure of the (XerCD–*dif*)$_2$ recombination complex. During, site-specific recombination by tyrosine recombinases, the two DNA strands are brought together in an almost planar configuration, with the four catalytic C-terminal domains of the recombinases on one side of the plane and the four N-terminal domains on the other side (Figure 8). As a consequence, the six FtsK subunits facing the (XerCD–*dif*)$_2$ complex are not equivalent: only half of them should be able to interact with the C-terminal catalytic domains of the XerD recombinases (Figure 8, bottom subunits). Thus, the nucleotides we identified in the vicinity of *dif* might reflect the DNA contacts made by only half of the FtsK translocation complex. Future experiments will aim at addressing as to what are the specific contacts made by FtsK on the sugar–phosphate backbone of DNA and by which amino acid residues these contacts are made.

SUPPLEMENTARY DATA

Supplementary Data is available at NAR Online.

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