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FtsK translocation on DNA stops at XerCD-*dif*

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ABSTRACT

***Escherichia coli* FtsK is a powerful, fast, double-stranded DNA translocase, which can strip proteins from DNA. FtsK acts in the late stages of chromosome segregation by facilitating sister chromosome unlinking at the division septum. KOPS-guided DNA translocation directs FtsK towards *dif*, located within the replication terminus region, *ter*, where FtsK activates XerCD site-specific recombination. Here we show that FtsK translocation stops specifically at XerCD-*dif*, thereby preventing removal of XerCD from *dif* and allowing activation of chromosome unlinking by recombination. Stoppage of translocation at XerCD-*dif* is accompanied by a reduction in FtsK ATPase and is not associated with FtsK dissociation from DNA. Specific stoppage at recombinase-DNA complexes does not require the FtsKγ regulatory subdomain, which interacts with XerD, and is not dependent on either recombinase-mediated DNA cleavage activity, or the formation of synaptic complexes.**

INTRODUCTION

Escherichia coli FtsK coordinates the late stages of cytokinesis and chromosome segregation. The 1329-amino-acid (aa) protein comprises three principal domains. A 279-residue N-terminal integral membrane domain functions in cytokinesis and localizes FtsK to the septum (1–4). A ~650-aa-long, proline- and glutamine-rich linker domain links the N-terminal domain to the ~500-aa-C-terminal DNA translocase domain that acts in the final stages of chromosome unlinking by chromosome dimer resolution and decatenation (5). The homohexameric FtsK translocase has three subdomains. FtsKαβ constitute the translocation motor, while the regulatory γ-subdomain, which is joined to the motor via a flexible ~20-aa linker, directs orientation-specific loading on DNA and activates chromosome unlinking through its specific interaction with the XerCD-*dif* recombination machine (6–8). FtsK translocation is rapid (~5 kb s^{-1} at 20°C), directional and has a high stall force (~60 pN) (9).

In vivo, the FtsK translocase appears to become active at the division septum late in cytokinesis, where it assembles on chromosomal DNA that has failed to segregate in a timely manner, because of (i) the presence of chromosome dimers that have formed by homologous recombination; or (ii) problems in chromosome decatenation; or (iii) delayed replication (10). FtsK translocation is guided by KOPS; DNA sequence consensus: GGGNAGGG (11,12), whose orientation is biased in each replichore, directing FtsK towards the XerCD-*dif* machinery located in the replication termination region, *ter*, where FtsK activates recombination through a specific interaction with the XerCD tyrosine recombinase bound to the recombination site *dif* (13).

Many helicases and translocases have been shown to strip proteins from DNA in order to mediate their normal DNA processing function e.g. Rep (14); UvrD (15); Srs2 (16); Dda (17); and chromatin-remodelling proteins (18,19). Furthermore, the *Bacillus subtilis* FtsK orthologue, SpoIIIE, efficiently strips proteins from chromosomal DNA as it is transported into the forespore (20). Here, we show that *in vitro* FtsK can remove proteins from DNA as it translocates. Nevertheless, FtsK translocation stops specifically on encountering XerCD-*dif* from either side, as would be expected from the biological role of FtsK in activation of XerCD-*dif* recombination. Stoppage does not apparently result in FtsK dissociation and is associated with a decrease in FtsK ATPase. In contrast, the EcoKI translocase displaces XerCD from *dif*. The stoppage reaction, which also occurs when FtsK encounters the tyrosine recombinase, Cre, bound to *loxP*, is not dependent on either the formation of recombinase–DNA covalent complexes, or on the formation of recombinase–DNA synaptic complexes.

MATERIALS AND METHODS

Proteins

His-tagged $FtsK_{50C}$ and its variants were purified as described (21). FtsKΔγ is $FtsK_{50C}$ deleted from residue

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1248, and FtsKΔ'handle' variants were derivatives of $FtsK_{50C}$ and FtsKΔγ deleted for residues 1095–1105. Proteins were dialysed into 20 mM Tris–Cl (pH 7.5), 250 mM NaCl, 10% (v/v) glycerol, 1 mM DTT after purification. XerC and XerD were purified as described (22).

DNA substrates for triplex displacement

Linear DNA substrates were derived from a 2.9-kb plasmid and carried a 20-nt triplex-binding site (TBS) 15 bp from one end (6) and a roadblock binding site: *dif*, *loxP* or their variants or *matS* 12 bp away from the TBS. Triplexes were formed by incubating 50 nM duplex DNA with 10–20 nM of a 5′ radio labelled triplex-forming oligonucleotide (TFO) in 10 mM MES (pH 5.5), 12.5 mM $MgCl_2$ at 20°C for ~16 h and were transferred to 4°C for ~4 h before use.

Triplex displacement assays

Reactions were performed at 21°C in 20 mM Tris–acetate (pH 7.5), 2 mM $Mg(OAc)_2$, 50 mM NaCl, 5% glycerol, 1 mM DTT (for FtsK) or 20 mM Tris–acetate (pH 7.9), 10 mM $Mg(OAc)_2$, 50 mM KOAc, 100 μM S-AdoMet, 1 mM DTT (for EcoKI). Triplex DNA (5 nM duplex) and roadblock protein were incubated for 2 min. Roadblock proteins were at saturating concentration for DNA binding (except for XerC, whose maximal soluble concentration gave ~50% binding); these were: XerC 125 nM, XerD 50 nM, MatP 20–100 nM, Cre 50 nM, Cre^{A36V} 150 nM, $XerC^{K172Q}$ 250 nM, $XerD^{K172Q}$ 100 nM. Binding of recombinases to DNA was assayed under the conditions for triplex displacement by mobility shift experiments during electrophoresis (22–25) and the concentrations of protein that gave ~50% DNA binding were similar to those published; >100 nM for XerC; ~10 nM for XerD and <1 nM for XerCD (23–25). FtsK derivatives were added at 750 nM (monomer); 50 nM EcoKI (D298E mutant) and incubated for a further 2 min. Reactions were initiated by adding ATP at 2 mM (4 mM for EcoKI). Twenty-microlitre aliquots were removed at 0 (before ATP addition), 1 and 2 min, quenched in buffer containing 5% (w/v) glucose, 1% (w/v) SDS, 80 mM MOPS (pH 5.5), 0.1 mg/ml bromophenol blue and assayed on a 6% polyacrylamide gel at 4°C containing TAM (40 mM Tris–acetate, 1 mM $MgCl_2$, pH 7.0). The TFO displacement values were normalized by subtracting unbound TFO and TFO displaced in the absence of FtsK. Reactions were normalized by subtracting unbound TFO at zero time and background displacement in the absence of FtsK. Displacement in the presence of roadblock was calculated as fraction of a displacement lacking roadblock (as percentage), and 'stoppage' calculated by subtracting these values from 100%. Reactions were performed mostly in triplicate.

ATPase assays

ATP hydrolysis rates were determined using an NADH coupled assay in 96-well plates (26). Reactions (200 μl/well) contained 5 nM short duplex DNA, 250 nM FtsK (monomer), 125/50 nM XerC/D, 25 mM Tris–acetate (pH 7.5), 10 mM $Mg(OAc)_2$, 50 mM NaCl, 5% glycerol, 1 mM DTT, 3 mM phosphoenolpyruvate, 0.8 mM NADH and 10 U/ml each of pyruvate kinase and lactate dehydrogenase. DNA and XerC/D were pre-incubated for ~2 min; followed by further incubation ~2 min with FtsK. The reactions were initiated by addition of 2 mM ATP and followed spectrophotometrically at 340 nm (±5 nm band-pass filter) using a BMG Labtech PHERAstar plate-reader fitted with a UV-vis absorbance module. Hydrolysis rates were determined from a linear fit to data averaged from three traces.

RESULTS

FtsK stops translocating at XerCD-*dif*

To assess whether FtsK can translocate through the XerCD-*dif* complex, we used triplex displacement as a readout of translocation (27). We positioned 28 bp *dif* 12 bp from a triplex oligonucleotide-binding site on a linear substrate and measured the proportion of TFO displaced, in the absence or presence of saturating concentrations of XerCD (Figure 1a; left panel). Incubation of the triplex substrate with FtsK and ATP resulted in 33.9 ± 1.2% and 36.9 ± 1.1% displacement of the TFO (±SEM, $n = 48$) within 1 min and 2 min after the initiation of translocation, respectively (Figure 1b).

Displacement was tested on two substrates bearing *dif* in either orientation. We refer to these orientations as 'XerD-first' or 'XerC-first', depending on which recombinase is encountered first by FtsK (Figure 1b). 'Stoppage' is used to describe the proportion of FtsK that fails to translocate through the potential block in triplex displacement reactions, as compared to control reactions in the absence of the block. The normalized values correct for any day-to-day variability in the specific activity of the FtsK protein. Zero prcent stoppage means displacement of TFO in the presence of the block is unchanged, as compared to the control, whilst 100% stoppage reflects a complete failure to displace the TFO in the presence of the block.

XerCD bound to *dif* reduced FtsK translocation dramatically, with no significant effect of orientation (79% and 83% stoppage for 'XerD-first' and 'XerC-first' orientations, respectively (Figure 1b). This is consistent with our expectation that *in vivo* FtsK can approach XerCD-*dif* from either side.

To determine whether the stoppage is specific to XerCD-*dif*, we substituted XerCD-*dif* by the specific DNA-binding protein MatP bound to *matS* (28). MatP-*matS* was chosen because this interaction occurs in the *ter* region of the *E. coli* chromosome, where FtsK translocation is known to occur. MatP was completely displaced, even at saturating concentrations of 100 nM, where stoppage was ~5% at 1 min (Figure 1c). MatP binds *matS* with moderate affinity (28) ($K_D \sim 10$ nM), comparable to that of XerD alone to *dif*. Furthermore, FtsK can efficiently displace streptavidin from biotin on DNA despite the femtomolar K_D of the streptavidin–biotin interaction (Graham,J.E., Sherratt,D.J., Crozat,E. and Howarth,M. unpublished data).

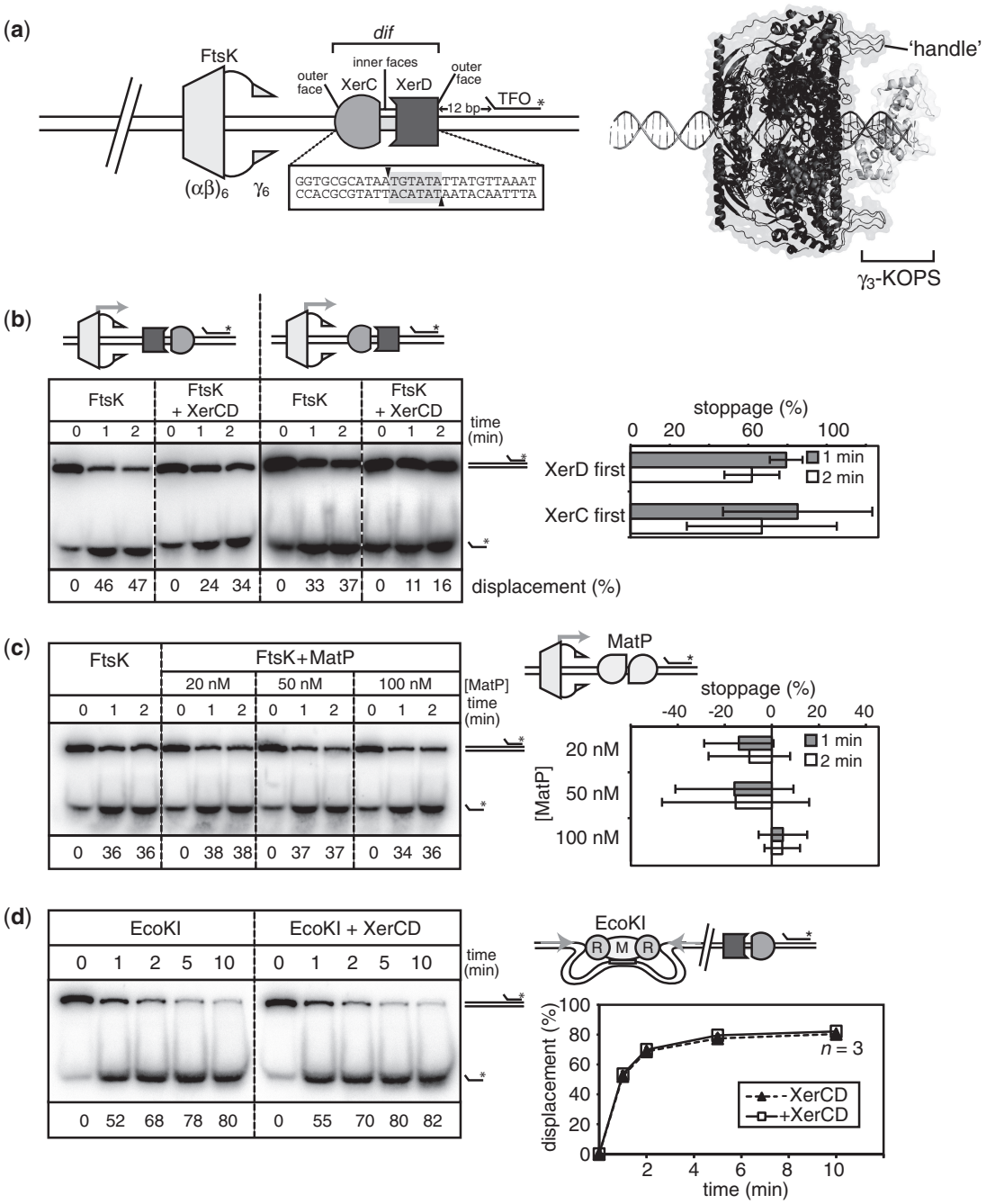


Figure 1. Stoppage of FtsK translocation by the XerCD-*dif*. (**a**) Left panel: Translocation was measured by displacement of a radio labelled TFO in the presence or absence of bound recombinases ('Materials and Methods' section). Substrates contained a triplex binding site biased towards one end of a linear DNA. The hexameric FtsK ring is represented as a cone with only two γ-subdomains shown for clarity. The inset shows the sequence of the *dif* site in 'XerC-first' orientation, XerC being the recombinase that would first be encountered by FtsK. Positions of the scissile phosphates in the recombination reaction are indicated with black triangles, and the central region with a grey box. We define the 'inner' face of XerC/D as that which faces the central region. (a) Right panel: Model of the hexameric FtsK motor loaded on KOPS DNA. This model is a composite of the crystal structures of the hexameric αβ-subdomains (30) and the trimeric γ-subdomains bound to KOPS (21) with B-DNA modelled through the DNA channel. The model represents an 'initiation complex' for FtsK translocation. The 'handle' of FtsK is also indicated. (**b**) The stoppage by XerCD was measured on substrates carrying the *dif* site in an 'XerD-first' or 'XerC-first' orientation (cartoons, left). Representative gels are shown. Time-courses (0, 1 and 2 min), with raw displacement values (with unbound TFO at zero time subtracted) from a single experiment, are shown beneath each gel. Bars show normalized stoppage values from three experiments expressed as a percentage of a control experiment lacking recombinase at 1 and 2 min (±SD) ('Materials and Methods' section). (**c**) MatP–*matS* complex does not stop FtsK translocation. Normalized stoppage values are shown, as per (b). (**d**) EcoKI displaces XerCD from *dif*. Translocation by EcoKI was investigated on the substrate containing *dif* in the 'XerD-first' orientation, with and without bound XerCD. Translocation initiates from the EcoKI site bound by the methyltransferase (rectangular box and 'M'), and the cartoon shows the looping of DNA by the translocating HsdR subunits (R). Triplex displacement profiles are shown for 10-min reactions performed in triplicate.

We also replaced FtsK with DNA translocase, EcoKI, a Type I restriction endonuclease that loads onto DNA at its recognition sequence and translocates bidirectionally in an ATP-dependent manner (29) (Figure 1d). Triplex displacement profiles over 10 min were virtually identical with and without XerCD bound adjacent to the TFO. Thus, unlike FtsK, EcoKI completely displaced XerCD bound to *dif*. Taken together, these observations suggest that FtsK translocation stoppage by XerCD-*dif* has specificity that relates to biological function.

XerD binding to a *dif* half-site blocks translocation

In order to understand the mechanistic nature of the stoppage, the interaction between FtsK and XerCD-*dif* was reduced to its minimal components by measuring triplex displacement when only one of the two recombinases was bound to *dif* or a *dif* half-site (Figure 2). In these experiments, FtsK encounters either the 'outer' or the 'inner' face of each recombinase. The 'outer' face is the surface normally encountered during FtsK translocation, while the 'inner' face of XerC or XerD normally constitutes the interface between the recombinases when they are bound to *dif*.

Strikingly, XerD was a powerful roadblock when FtsK encountered its outer face, but not when it was approached on its inner face, again emphasizing specificity in the stoppage reaction (Figure 2; compare i with iii). We believe that the only ~2-fold difference in stoppage between the two directions of approach reflects the fact that XerD can also bind the XerC-binding site of *dif* non-cooperatively and with relatively low affinity (23,24) and that much of the stoppage observed in the 'inner face' approach experiment actually results from stoppage at the outer face of a second bound XerD molecule. Indeed at the XerD concentrations used, a significant amount of the $XerD_2$ complex was observed in mobility shift electrophoresis assays (data not shown). Therefore, we repeated the experiments using *dif* half-sites, which only bind a single recombinase molecule (Figure 2, v–viii). As anticipated, the results show a stronger discrimination in stoppage (~5-fold) on approach of XerD from the outer face as compared to the inner face, thereby supporting the conclusion of specificity in orientation-dependent stoppage by XerD binding to *dif*. The reduced stoppage of FtsK translocation by the outer face of XerD alone as compared to XerCD is likely due to the shorter half-life of XerD-*dif* complexes as compared to XerCD-*dif* complexes (below).

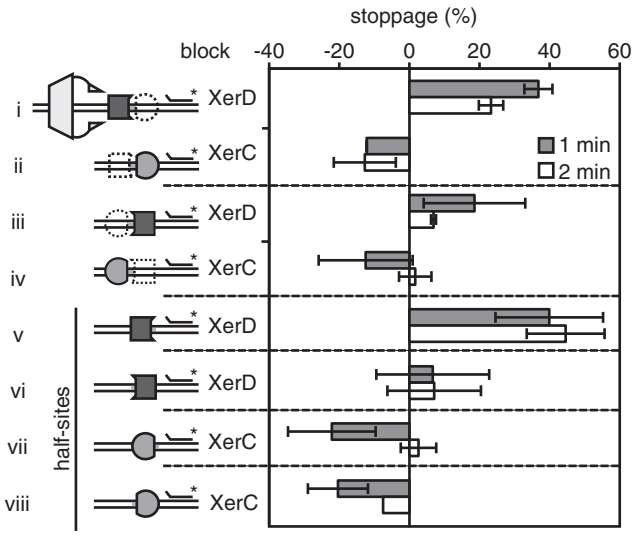


Figure 2. Stoppage of translocation by XerC and XerD recombinases bound alone to their cognate *dif* half sites. Triplex displacement by FtsK was measured on substrates bearing a complete *dif* site but with only one of the Xer recombinases bound (**i–iv**), or on half *dif* sites containing only 2 bp of the central region (**v–viii**). The composition and orientation of these sites is shown schematically. Normalized displacement values are expressed as per Figure 1.

XerC did not apparently pose a block to translocation in either orientation (Figure 2; ii and iv). This is likely due to the poor affinity of XerC alone for its *dif* binding site ($K_D > 100$ nM), when compared to XerD ($K_D \sim 10$ nM) and the highly cooperative binding of XerCD to *dif* ($K_D < 1$ nM) (25–27). This is expected to result in a half-life of seconds on *dif* DNA as compared to a few minutes for XerD and many minutes for XerCD complexes, assuming a diffusion determined k_{on} of $\sim 10^7\ M^{-1}\ s^{-1}$.

FtsKγ is not required for stoppage

The FtsK γ-subdomain interacts directly with the C-terminus of XerD to activate XerCD-*dif* recombination (13). It therefore seemed possible that this interaction might be important in translocation stoppage. Surprisingly, XerCD-*dif* stopped FtsK deleted for the whole γ-subdomain efficiently in both orientations (Figure 3). Thus, the ability of XerCD-*dif* to stop FtsK translocation does not require the XerD–FtsKγ interaction. Consistent with this result, an XerD mutant ($XerD^Q$) impaired in its interaction with the FtsK γ-subdomain (13) also stopped FtsK and FtsKΔγ translocation. Our failure to see significant discrimination in FtsKΔγ stoppage as a consequence of direction of approach when XerD alone was present (Figure 3; second panel from top) is almost certainly a consequence of two XerD molecules binding to the *dif* site; on a *dif* half-site, we observed an ~3-fold greater stoppage on approach from the outer face as compared to the inner face (data not shown).

In search for another structural feature in FtsKαβ that could potentially be involved in stoppage we focused on a protruding 'handle' (30), which is the most obvious structural feature on the leading face of the motor domain (residues 1095–1105; Figure 1a, right panel), and which is not required for translocation (below). A loop similar to the FtsK handle is implicated in a regulatory activity of PspF, a related protein, responsible for σ^{54}-factor remodelling (31). FtsKΔ'handle' variants, with and without the γ-subdomain, stopped at XerCD-*dif*, just like FtsK. After 1 min, FtsKΔ'handle'Δγ showed 76% and 89% stoppage in the 'XerC-first' and 'XerD-first' orientations (SD ± 8%; ±6.5%), respectively; while FtsKΔ'handle' showed 70% and 40% stoppage in the same orientations (SD ± 32%; ±10%), respectively.

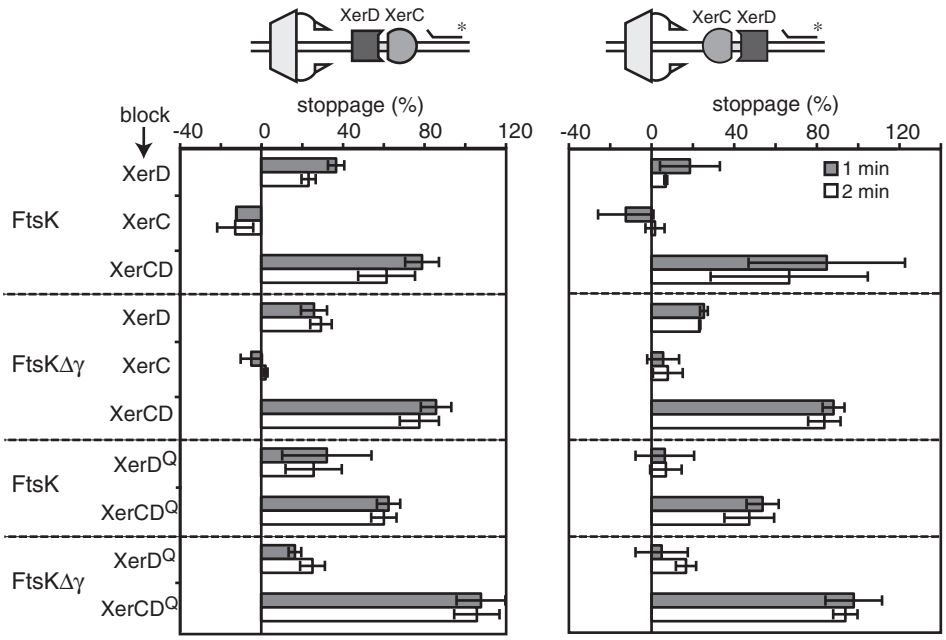


Figure 3. Specific FtsKγ-XerD interaction is not required for FtsK translocation stoppage. Triplex displacement by FtsK and FtsKΔγ was measured on a substrate with *dif* in either orientation (cartoons), and in the presence of XerC and/or XerD recombinases, or a quadruple mutant of XerD (R288K Q289R Q292E Q293R), XerD^{Q}.

Cre-*loxP* blocks FtsK translocation

To address further the issue of specificity of FtsK stoppage, we tested whether the related tyrosine recombinase, Cre, bound to its site *loxP* (32,33), stopped translocation. The 34-bp *loxP* site contains a 6-bp central region flanked by two imperfectly symmetrical Cre-binding sites. Synapsis-dependent cleavage occurs preferentially on the 'bottom'-strand first as a consequence of different configurations of the two Cre monomers bound to a *loxP* site (32). Unlike XerCD–*dif*, Cre–*loxP* recombination does not require FtsK for activation, therefore, we would not, *a priori*, expect a specific interaction, similar to the one between FtsKγ and XerD to be occurring with Cre. However, FtsK has been shown to simplify the topology of Cre–*loxP* recombination products on plasmid substrates *in vitro* (34) and does allow Cre–*loxP* to substitute for XerCD–*dif* in mediating chromosome dimer resolution *in vivo* (35,36).

Under conditions where Cre saturates *loxP*, the Cre–*loxP* complex stopped FtsK translocation (Figure 4). Strikingly, there was a clear dependence on *loxP* orientation, such that when FtsK approached Cre–*loxP* complex from the side that is preferentially cleaved, 'P', stoppage was efficient (Figure 4, 'P'). Encounter from the other side resulted in little or no stoppage ('NP'), suggesting that either a difference in Cre monomer 'presentation' to FtsK or a local protein/DNA configuration on this side of the complex was important in the stoppage reaction. Cre bound to a half-'P' site, oriented such that FtsK would encounter its outer face first, showed 42% stoppage in a 1-min reaction, but when encountered on the inner face showed 13% stoppage, once again demonstrating specificity in the stoppage reaction

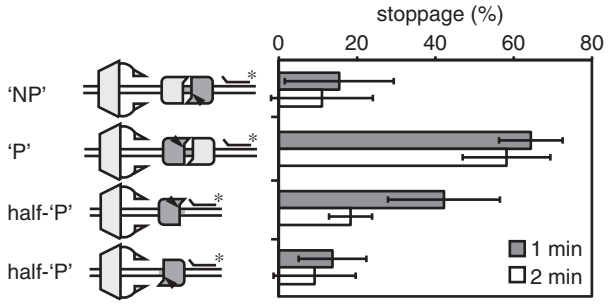


Figure 4. Translocation stoppage at Cre recombinase bound to *loxP*. The ability of FtsK to displace Cre from *loxP* was measured on substrates with *loxP* in either orientation. The orientation of *loxP* is defined by the asymmetry of the nucleotide sequence close to the scissile phosphate, which results in preferential cleavage of one scissile phosphate (black arrow; ref. 32). We denote the central region and define the inner and outer faces of Cre in the same manner as in Figure 1a. The Cre monomer that mediates the first cleavage is indicated by a darker shade of grey. 'P-first' and 'NP-first' denote the orientations of *loxP* in which FtsK approaches preferentially and non-preferentially cleaved half sites of the *loxP* first, respectively. We also tested displacement on a substrate carrying a half-*loxP* site that contains the preferentially cleaved scissile phosphate. This half-site was also tested in both orientations, so that FtsK approached the Cre monomer either on its inner or outer face.

(Figure 4). Similarly to XerD, a Cre monomer bound to *loxP* half-site acted as a weaker roadblock than the Cre–*loxP* complex in the same orientation, perhaps because of lower occupation of the site by a single Cre monomer in the absence of Cre-binding cooperativity, consistent with the observed decreased stoppage in a 2-min reaction as compared to a 1-min reaction.

These results suggest that stoppage of FtsK translocation by tyrosine recombinases bound to their

recombination sites may be a general phenomenon that relates to the *in vivo* biology of these recombinases and to FtsK.

Stoppage does not require recombinase-mediated DNA cleavage or synapsis of recombination sites

In order to reveal the mechanism by which tyrosine recombinase–DNA complexes stop FtsK translocation, we tested whether synaptic complexes of two recombination sites and recombinase tetramers, and/or recombinase–DNA covalent intermediates were acting as the roadblocks. To address this we used a Cre^{A36V} variant which binds *loxP* sites with an affinity similar to wild type Cre, but is deficient in synapsis (37). As a consequence of being impaired in synapsis, this mutated protein has also greatly reduced DNA cleavage activity (27). Under saturating conditions, Cre^{A36V} bound to the *loxP* in orientation 'P', the more effective orientation for stoppage, was able to stop FtsK translocation efficiently, with no significant difference as compared to wild-type Cre, indicating that neither synapsis nor DNA cleavage is a prerequisite for stoppage (Figure 5a).

$XerC^{K172Q}$ and $XerD^{K172Q}$, mutated for a conserved active site lysine are defective in DNA cleavage (Cao,Y., Graham,J.E., Sherratt,D.J. and Arciszewska,L.K. unpublished data). These variants are equivalent to Cre^{K201A} which is also deficient in cleavage but proficient in synapsis (37,38). $XerC^{K172Q}$ and $XerD^{K172Q}$ either in combination with the wild-type partner recombinase, or with each other, stopped FtsK translocation on the 'XerD-first' *dif* substrate (Figure 5b). This reinforces the conclusion that a phosphotyrosine covalent intermediate is not involved in stoppage. Taken together, the results show that neither phosphotyrosine intermediates nor synaptic complexes are required for FtsK translocation stoppage.

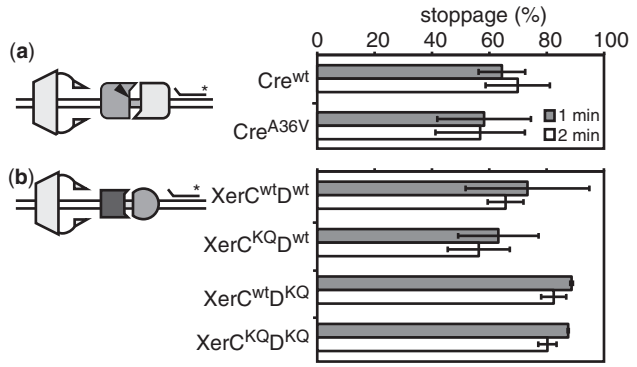
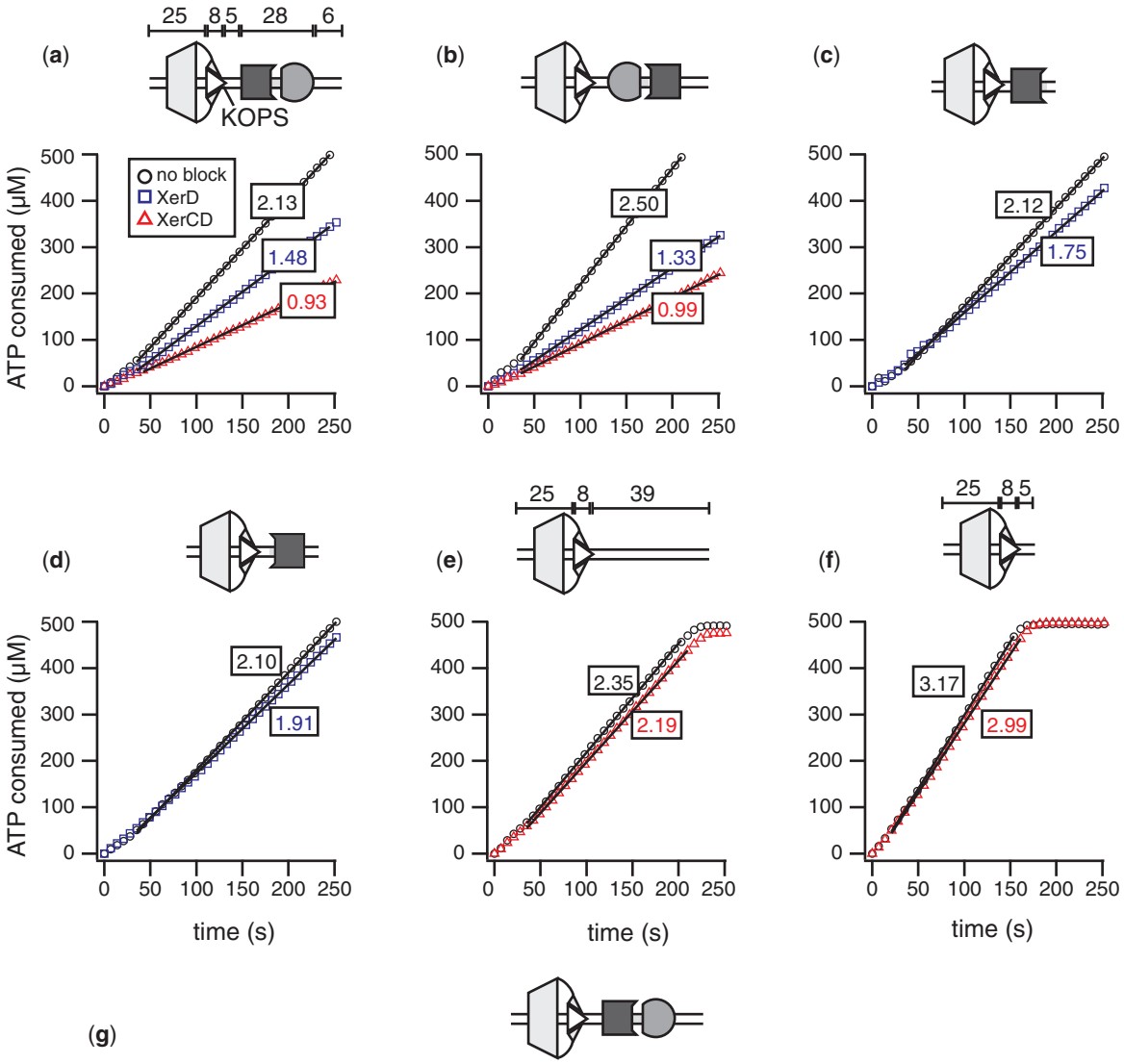


Figure 5. Recombinases deficient in DNA synapsis or cleavage stop translocation of FtsK. (**a**) Triplex displacement was measured on a substrate bearing a *loxP* site with the active Cre monomer first (orientation 'P', cartoon), in the presence of either wild-type Cre or a synapsis-defective Cre mutant (A36V). (**b**) Normalized triplex displacement by FtsK on a *dif* substrate in the presence of combinations of wild-type XerC, XerD and catalytic mutants $XerC^{K172Q}$ and $XerD^{K172Q}$.

Stoppage is accompanied by a reduction in FtsK ATPase activity

To elucidate further the stoppage mechanism, we analysed FtsK ATPase activity upon encountering the roadblock. We used short linear DNA substrates, 25-KOPS-5-*dif*-6 and 25-KOPS-5-*revdif*-6, both carrying a KOPS FtsK loading site positioned 25 bp away from one end and *dif* site in either 'D-first' or 'C-first' orientation located 5 bp away from KOPS (Figure 6a). The expectation was that in the absence of XerCD, the steady-state FtsK ATPase activity reflected cycles of protein loading at KOPS followed by translocation over a distance of 47 bp and dissociation/stalling/treadmilling at the end of the DNA fragment. In the presence of XerCD, FtsK translocation will stop after 5–13 bp, thereby preventing loading of a second hexamer at KOPS, unless the stopped hexamer dissociates from DNA. Therefore, if the stopped hexamer remains associated with DNA and has its ATPase downregulated, a substantial decrease in ATPase would be expected in the steady-state assays. In contrast, close-to-normal ATPase levels would be expected if stoppage were accompanied by continuing non-productive ATP hydrolysis. If translocation stoppage were a consequence of FtsK dissociation after encountering XerCD-*dif*, further cycles of FtsK loadings and short translocations might also be expected to give close-to-normal levels of ATPase.

ATPase decreased to 40–44% of its original value in the presence of XerCD bound to *dif* in either orientation (Figure 6a and b). No difference in ATPase activity was observed when FtsK was loaded in the absence or presence of XerCD on the same length substrate lacking *dif*, thereby demonstrating that the decreased ATPase is a direct consequence of translocation stoppage at XerCD-*dif* (Figure 6e). On a shorter substrate lacking *dif*, in which translocation could only occur over 5–13 bp (25-KOPS-5; Figure 6f), a higher level of ATPase was observed than with the longer substrates, confirming that cycles of FtsK loading and translocation can lead to robust ATPase and that the reduced ATPase levels observed in the presence of XerCD-*dif* are likely the consequence of ATPase downregulation, rather than a reduced distance available for translocation. The data also support the conclusion that the stopped FtsK does not dissociate when it encounters XerCD-*dif*, since if it did, cycles of FtsK and short-distance translocation could occur, resulting in substantial ATPase levels, although these might still be lower than on a short protein-free DNA fragment on which non-productive ATP hydrolysis might occur on reaching the DNA end. Since translocation stoppage at XerCD-*dif* is only ~81% efficient, when judged by triplex displacement (Figure 1b), most or all of the residual ATPase measured in the presence of the XerCD-*dif* substrate can be accounted for by FtsK translocation through *dif*, either because XerCD was transiently unbound to *dif*, or because translocation occasionally removed the roadblock; compare the $0.93\,\mu M\,s^{-1}$ ATPase in the presence of XerCD-*dif* (Figure 6a) and the $3.17\,\mu M\,s^{-1}$ ATPase on the short substrate (Figure 6f).



FtsK variant	Concentration (nM monomer)	ATPase activity (μM / s)		% reduction in ATPase
		−XerCD	+XerCD	
wild-type FtsK	250	2.13	0.93	56
FtsKΔγ	250	0.61	0.27	56
FtsKΔγ	500	1.73	0.70	60
FtsKΔhandle	250	0.88	0.42	52
FtsKΔhandleΔγ	250	0.86	0.43	50

Figure 6. FtsK ATPase activity is reduced upon stoppage by XerCD-*dif*. The ATPase activity of FtsK was measured on short linear substrates bearing a KOPS loading site with a 25-bp loading arm, and *dif* site positioned 5 bp away from the KOPS (cartoons) using an NADH coupled assay. Loading on KOPS directs FtsK translocation towards *dif*, as indicated by the arrow. The experiments were performed with substrates bearing a full *dif* site in XerD-first (**a**) or XerC-first (**b**) orientation and on an XerD *dif* half-site in both orientations (**c**) (**d**) and substrates lacking *dif* and carrying either a 39 bp (**e**) or 5 bp (**f**) extension downstream of KOPS. The traces begin ~30 s after the addition of 2 mM ATP. ATPase rates (μM/s) are shown in boxes next to each graph; they were determined from linear fits to the average of three traces; SDs were <0.05 (μM/s). (**g**) ATPase activity of FtsK variants on a short substrate carrying *dif* in XerD-first orientation [as in (a)].

Note that the somewhat lower ATPase activity measured on the longer substrates in the absence of XerCD, when compared to the 25-KOPS-5 substrate, could be because KOPS-independent FtsK loadings are more frequent on the longer substrates, resulting in collision with KOPS-loaded hexamers and consequent inhibition of translocation. Such events would not occur in the longer substrates in the presence of XerCD.

The presence of XerD bound to half site *dif* led to a small decrease in ATPase when FtsK encountered on the outer face (18%; Figure 6c). The relatively small decrease in ATPase activity as compared to the greater stoppage

values by XerD in the TFO displacement assay may be attributed to a less-stable XerD binding close to the end of the short DNA substrate used in the ATPase assay. A smaller decrease in ATPase activity was observed when FtsK encountered the XerD inner face (9% reduction; d), consistent with the triplex displacement data.

XerCD-mediated stoppage of FtsKΔγ, FtsKΔ'handle' and FtsKΔ'handle'Δγ on the 25-KOPS-5-*dif*-6 substrate, resulted in similar decreases in the levels of ATPase activity as stoppage of FtsK (Figure 6g), reinforcing the conclusion that neither the γ-subdomain, nor the 'handle', are involved in stoppage and associated downregulation of the motor. Taken together, we conclude that it is likely that FtsK encounter with XerCD-*dif* leads to specific downregulation of FtsK ATPase, without translocase dissociation, therefore leaving FtsK close to XerCD in order to ensure XerCD-*dif* recombination. Nevertheless, we cannot absolutely eliminate the possibility that stoppage is associated with FtsK dissociation, although this would require activation of XerCD recombination before dissociation.

DISCUSSION

The work presented here details the inability of a fast, powerful dsDNA translocase to remove a tyrosine recombinase complex from its cognate binding site. We have ruled out the involvement of synapsis, covalent DNA–recombinase complexes and specific interaction between the FtsKγ-subdomain and the recombinases in the stoppage reaction. Nevertheless, there is specificity in the stoppage reaction because FtsK can displace other proteins from DNA, while XerCD can be displaced by the EcoKI translocase. Furthermore, FtsK only stops when it encounters the outer face of the recombinase. These observations relate directly to the major *in vivo* function of FtsK translocase, which is to activate chromosome unlinking by XerCD-*dif* recombination. If FtsK could displace XerCD from *dif*, recombination would not be possible. The observation that FtsK also stops at *loxP*-bound Cre suggests that FtsK can access P1 DNA prior to its segregation in the plasmid prophage state and that it may function in the simplification of P1 plasmid topology during unlinking, just as FtsK translocation simplifies topology during XerCD-mediated unlinking (7). Relevant to this, the P1 prophage genome has KOPS sites oriented so that FtsK translocation will be directed towards to *loxP* site (39).

The demonstration that encounter of FtsK with the recombinase–DNA complex leads to an apparent downregulation of the FtsK motor, which remains associated with the XerCD-*dif* complex, is consistent with the biological role of FtsK in activating XerCD-*dif* recombination. Deletion of a protruding 'handle' in the αβ motor, and/or the γ-subdomain, two obvious FtsK features that might have been involved in the specific FtsK-recombinase interaction that leads to ATPase downregulation, did not impair stoppage. Plausible ways of motor down-regulation would be by disrupting monomer–monomer interactions in the homohexamer, since inter-subunit interactions generally play a key catalytic role in hexameric translocases and helicases (40). However, any such disruption appears not to lead to FtsK dissociation from DNA when it encounters XerCD–*dif*, since we have found no evidence for such dissociation, and dissociation would appear incompatible with XerCD–*dif* activation. Nevertheless, less drastic, but presumably cooperative, changes to inter-subunit interactions could lead to catalytic downregulation. For example, changes in the positioning of the putative arginine finger, which is expected to act *in trans*, from a neighbouring subunit to fulfil its role in catalysis (41). Other possible catalytic switches that could lead to motor downregulation include the 'glutamate switch' that controls the positioning of the catalytic glutamate in the Walker B DEXX motif in many AAA^+ ATPases (42).

We initially considered whether encounter of FtsK with the recombinase complex might induce the recombinase to clamp more tightly around DNA. Such a mechanism has a precedent with Tus-*terB*, where approach of the DnaB replicative helicase from only one direction is proposed to lock the complex via a base-flipping mechanism (43). In this case, there would be no need for a specific downregulation of ATPase activity, as shown here. Note that the Tus-*terB* stalling of replication appears to be induced by the specific change in DNA conformation at the replication fork. There is no evidence that a changed or unusual DNA conformation plays any role in translocation stoppage at a XerCD–*dif* complex, consistent with the observation that FtsK translocation can readily displace both Holliday junctions and 3-way T-junctions (11) (and Massey, T. and Sherratt,D.J, unpublished data). Nevertheless, the orientation-dependence of Cre stoppage at *loxP* that we observed is consistent with DNA conformation, associated with recombinase binding, being able to influence the stoppage reaction.

The FtsK orthologue, SpoIIIE, has shown to strip proteins from DNA as it transports a *B. subtilis* chromosome into the forespore during sporulation. This has been suggested to reset the epigenetic state of spore-specific DNA, which is crucial for the necessary differential gene expression profiles of forespore and mother cell (20). It seems certain that FtsK, will also strip proteins from DNA *in vivo*, as it does *in vitro*. Whether this has any implications for *E. coli* biology remains unclear, although we note that FtsK action is controlled spatially and temporally, being restricted normally to ~400 kb in the region of replication termination that contains *dif* and only occurs late in cytokinesis, when chromosome replication–segregation is likely to be complete in most cells (10) (and Pages,C. and Cornet,F., personal communication). The stoppage of FtsK translocation at XerCD-*dif* is not only essential if FtsK is to activate chromosome unlinking, but may help to minimize collisions with replication forks converging on *dif*, which could lead to replication fork reversal, unwanted homologous recombination and dsDNA breaks.

Although many helicases/translocases are able to displace DNA binding proteins as they move along

DNA, either non-specifically [e.g. Rrm3 (44); Rep (14); Dda (17)], or as a consequence of specific interactions (45,46), FtsK has the additional property that it stops translocation specifically at nucleoprotein recombination complexes, whose removal prior to the recombination reaction would be detrimental to the organism's biology. It seems certain that other translocases will display the similar property of motor downregulation (or dissociation) on encountering a specific nucleoprotein complex, followed by modification of the complex's activity.

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